IN VITRO AND *IN VIVO* ANALYSIS OF DIFFERENTIAL GENE EXPRESSION BETWEEN NORMAL NORFOLK TERRIER DOGS AND THOSE WITH AN AUTOSOMAL RECESSIVE MUTATION IN *KRT10*

A Dissertation

by

KIRSTIN FAYE BARNHART

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Veterinary Pathology

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ABSTRACT

In Vitro and *In Vivo* Analysis of Differential Gene Expression between Normal Norfolk Terrier Dogs and Those Affected with an Autosomal Recessive Mutation in *KRT10*.

(August 2004)

Kirstin Faye Barnhart, B.S, Texas A&M University;

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Dr. Kelly M. Credille

Natural diseases caused by keratin mutations are rare and have only been reported in humans. We have recently identified a heritable skin disorder in Norfolk terriers caused by a mutation in *KRT10*. Affected dogs have a tendency to form shallow erosions or blisters following mild trauma, which is first noted after the birthing process. As the dogs age, they display generalized hyperpigmentation and scaling that is most severe in the axillary and inguinal regions. The main histologic and ultrastructural features include: marked hyperkeratosis, epidermal hyperplasia, prominent vacuolation of the upper suprabasal layers, eosinophilic intracytoplasmic aggregates (keratin bundles), numerous and frequently enlarged keratohyaline granules, and epidermal hyperplasia. Analysis of an extended pedigree through seven generations confirmed an autosomal recessive mode of inheritance. The keratin 10 mutation was defined as a G-T point mutation in intron 5 that affected splicing at the boundary of exon 4 and intron 5. The primary outcome of the mutation was a 35 bp deletion in exon 4 caused by use of a

cryptic splice site. Real-time PCR quantitation of KRT10 confirmed that this mutation led to premature mRNA decay and an average 35-fold decrease in KRT10 message.

Organotypic cell culture techniques were used to establish *in vitro* models for normal and affected Norfolk terriers. After 21 days of culture, normal epidermis was cornified with a compact and multifocally parakeratotic stratum corneum. Affected epidermis largely reproduced the expected morphologic alterations. Immunoblotting and immunohistochemistry for keratin 10 protein and real-time PCR quantitation of *KRT10* message showed significantly less keratin expression *in vitro* than *in vivo* suggesting that the differentiation program *in vitro* underwent significant alterations.

A diagnostic PCR assay was established for detection of the carrier state. Global analysis of gene expression between normal, carrier and affected dogs was performed with DermArray cDNA microarrays. Affected and carrier dogs showed differential regulation of 320 and 298 genes, respectively, between normal dogs. In affected dogs, 217 were upregulated and 103 were downregulated. In carrier dogs, 222 were upregulated and 76 were downregulated. 72 genes (65 upregulated, 7 downregulated) were altered in both affected and heterozygous dogs.

For Trey
For Austin
and for Spook.

ACKNOWLEDGMENTS

I cannot fully express the gratitude I feel toward Kelly Credille and Bob Dunstan for seeing me through the last 6 years and supporting me in all endeavors - not only my pursuit of a doctoral degree, but also my desire to have a family and obtain board certification in the field of clinical pathology. They have been the most amazingly supportive colleagues, mentors and most importantly friends. To whatever heights the attainment of this final degree will take me, I owe a huge debt of gratitude toward them and their unfailing support.

This project would never have enjoyed even the slightest bit of success without the support and generosity of the Norfolk terrier breeders: Marlene Grief, Carol Faulk, Tony Harold, Tina Dennis and Patricia Rogers. They epitomize the utmost in character and integrity in their desire to breed Norfolk terriers that not only display desirable qualities in the show ring, but also maintain the highest quality of health possible. They have remained completely devoted to characterizing this skin disease and developing tests that prevent breeding of affected animals. They have allowed their dogs to be biopsied numerous times to provide the tissue necessary to define the disease. Should all breeders be so committed to the well-being of their dogs, heritable genetic defects would quickly become a thing of the past.

To the Norfolk terrier dogs: Bob, Haley, Rolex, Pickpocket, Austin, Kensington, Micki, Houston, Sprinkle, Seth, Kristi, Glory and Sweet Pea. Thank you so much for your delightful personalities and your kindness and patience during the biopsy

procedures. You represent a wonderful dog breed that will provide love and companionship to many people and families in the years to come.

And finally, I must thank my patient and loving husband who has tolerated countless late nights and weekends when I have had to remove myself from the normal activities of family life to write and write and write... Thank goodness only one of us feels the need to endlessly pursue education while foregoing financial security and sensible work hours. Hopefully, the outcome of this quest for a greater understanding of the pathologic basis of disease will benefit both human and veterinary medicine as I prepare to embark upon a career defining and developing therapeutic for cancer.

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CHAPTER I

INTRODUCTION

Overview of cornification

The skin's major structural barrier is provided by a few layers of keratinocytes that collectively comprise the epidermis. This barrier, known as the stratum corneum, is life-sustaining, prevents loss of water from the body and withstands chemical, microbial, immunological and ultraviolet/solar assault (Blumenberg and Tomic-Canic, 1997; Fuchs, 2001). The synthesis of the stratum corneum is often referred to as keratinization, but because this process involves more than keratins alone, cornification is the most complete term (Piérard *et al*, 2000).

Cornification represents the convergence of three biochemical processes, followed by desquamation: 1) the formation and organization of intracellular keratin intermediate filaments; 2) the formation and dispersion of an intercellular lipid glue or mortar that anneals the fully cornified cells together and makes the stratum corneum impermeable to many substances; and 3) the synthesis of the cornified envelope, the toughest portion of the stratum corneum that also functions to interconnect the intracellular keratin matrix with the intercellular lipid glue. Once keratinocytes have undergone complete cornification, they are called corneocytes (Piérard *et al*, 2000).

This dissertation follows the style and format of the Journal of Investigative Dermatology.

Keratin biology

Keratins form the type I and type II groups of the intermediate filaments, a superfamily of structural proteins that comprise one of the 3 main networks that form the cytoskeleton of eukaryotic cells. The other two components of the cytoskeleton, microfilaments (5-7nm diameter) and microtubules (approx 25 nm diameter) are primarily involved with cell division, contraction, polarization, orientation and anchorage. In contrast, intermediate filaments are primarily responsible for providing the structural support that allows cells to resist ongoing stress and trauma (Albers, 1996; Smith, 2003).

Six types of intermediate filaments (I-VI) have currently been identified. The type I and type II intermediate filaments are the keratins. They specifically confer structural stability to epithelial cells; however, recent studies have uncovered additional functions for keratin molecules that includes cell signaling (Kirfel *et al*, 2003; Paramio and Jorcano, 2002) and apoptosis (Dinsdale *et al*, 2004) and organization of cellular organelles (Kumemura *et al*, 2004)

As with all intermediate filaments, keratins share a basic homologous structure that consists of a 310 amino acid domain with an amino-terminal ("head"), a carboxy-terminal ("tail") and an a-helical central rod with four separate helices (1A, 2A, 1B, and 2B) that are separated by the non-helical linkers (L1, L12, and L2) that confer flexibility to the molecule. The terminal regions are generally divided into 3 subdomains: the H (homologous) domain, the V (variable) domain and the E (end) domain.

The a-helical region is extremely conserved in size and function, and the amino acids are arranged in heptad repeats $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ with hydrophobic residues located at the *a* and *d* positions that ultimately associate to form a left-handed coiled-coil polypeptide. The other positions generally contain hydrophilic residues, which help determine higher orders of intermediate filament assembly. The heptad repeats reverse in the middle of the 2B rod domain creating a region termed the "stutter" region, which is important to the structural stability of intermediate filaments possibly by conferring flexibility (Brown *et al*, 1996) or promoting filament elongation (Hermann *et al*, 1999). Recent studies have indicated that the *a* and *d* heptad positions form a "knob-in-hole" backbone for the a-helices. Although keratins lack the traditional trigger motifs found in other coiled-coil molecules, several candidate 13 base pair trigger motifs that may also be important for coiled-coil formation have been identified. These coiled-coil trigger motifs are required for the stability of keratin intermediate filaments (KIF) (Wu *et al*, 2000). (Fig 1). Two short regions are present at the end of each rod domain: the helix initiation motif and the helix termination motif. Both of these regions are highly conserved and believed to confer added stability during filament assembly (Steinert *et al*, 1993) The type II keratins have two additional subdomains, H1 and H2 located between the rod domain and the V1 and V2 domain, respectively (Smith, 2003). Type I keratins do not contain a homologous (H) region and for this reason, are smaller than type II keratins. (Steinert, 1993). Many of the amino acid sequences located throughout the central rod domain of the intermediate filament proteins are highly conserved. The most variable sequences reside in the head and tail end regions.

Type I and type II intermediate filaments are the acidic and basic keratins, respectively which are obligate heterodimers that assemble to form a complete 10-12nm KIF. (Steinert, 1990). In most cases, this binding is specific, i.e. a unique type I keratin pairs with a unique type II keratin (Steinert, 1993). Specific keratin pairs are expressed in a tissue-specific and differentiation specific manner (Moll, 1982), and cutaneous keratin pairs are expressed in different levels of the epidermis. The normal basal layer

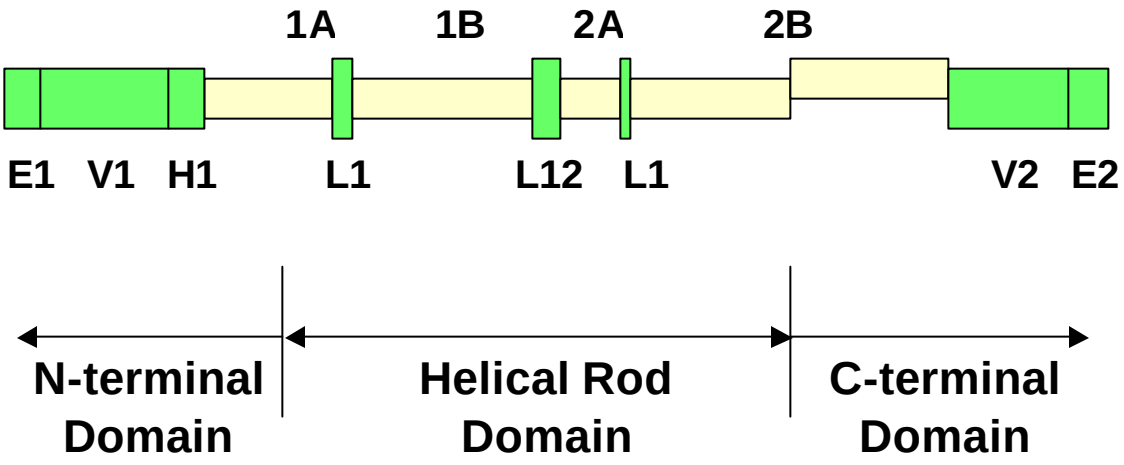

Figure 1. Structure of a type II keratin.

Contains keratins 5 and 14 while keratins 1, 2e and 10 predominate in the normal suprabasal epidermis and stratum corneum (Steinert *et al*, 1993).

To date, 49 different keratins (25 type I and 24 type II) have been identified (Coulombe and Omary, 2002). Type I and type II keratins were initially distinguished based on sequence similarities, but they also have distinctive biochemical properties that set them apart. Type I keratins in addition to being smaller (approximately 40-57 kDa), have an acidic pH and are located on chromosome HS17q12-q21. Type II keratins are larger (approximately 52-67 kDa), have a neutral or basic pH and are located on chromosome HS12q11q-14 in humans (Mischke, 1998; Chu and Weiss, 2002). Keratin molecules are assembled into coiled-coil heterodimers such that the a-helical domains are parallel and in exact axial register. This dimerization occurs spontaneously without the assistance of additional proteins and forms bundles or ropes of a-helices that define the tertiary conformation of the keratin molecules.

Once this initial dimerization has occurred, the next step in KIF formation is the alignment of nearest-neighbor molecules to form a tetramer. This precise arrangement has been controversial and is still uncertain. According to his model (Steinert *et al*, 1993), the alignment of the keratin dimers to form tetramers occurs in three different antiparallel orientations also called modes (A_{12}, A_{11} and A_{22}) that are defined by which portions of the rod domains partially or fully overlap. A_{12} mode occurs when two dimerized molecules are antiparallel and in almost perfect alignment. A_{11} is a staggered mode in which these molecules are staggered so that the 1B segments are closely aligned. Lastly, the A_{22} mode is also a staggered mode, but instead of the 1B segments,

the 2B segments are closely aligned. These modes are stable under different pH values; however, the method by which these different orientation modes are stabilized *in vivo* is not clear. The presumption is that 8-16 molecule-wide particles form a stacked array of alternating rows of either A_{12}/A_{11} or A_{11}/A_{22} molecules. A fourth mode, I, occurs at higher levels of association. I is not an antiparallel arrangement, but a similarly directed mode in which the last 8-10 residues of the 2B segment of one molecule overlap with the first 8-10 residues of the next molecule in the column. These modes may then be further stabilized by intermolecular sulfide bonds.

The alignment of these modes helps define the quaternary structure of keratin proteins. Based on these alignments, a polymer lattice is formed and two dimensional diagrams can be created that show the organization of keratin molecules within the latticework. Mass measurements have indicated that keratin intermediate filaments contain on average 16 molecules (molecule = keratin dimer). They contain equal numbers of alternating in-register and staggered molecules that generate a characteristic axial repeat of 22.6nm. These aligned chains contain five areas of overlapping sequences. Interestingly, these overlapping sequences are the most highly conserved in the intermediate filament superfamily (Steinert *et al*, 1994).

General terminology applied to the quaternary structure of keratin filaments has not always been consistent. Currently, the most widely used terms are protofilaments and protofibrils. Protofilaments are defined as long subfilamentous structures that in cross-section contain two type I/II molecules (four chains) and have a diameter of approximately 3 nm. The exact structure of protofibrils still requires verification, but it is

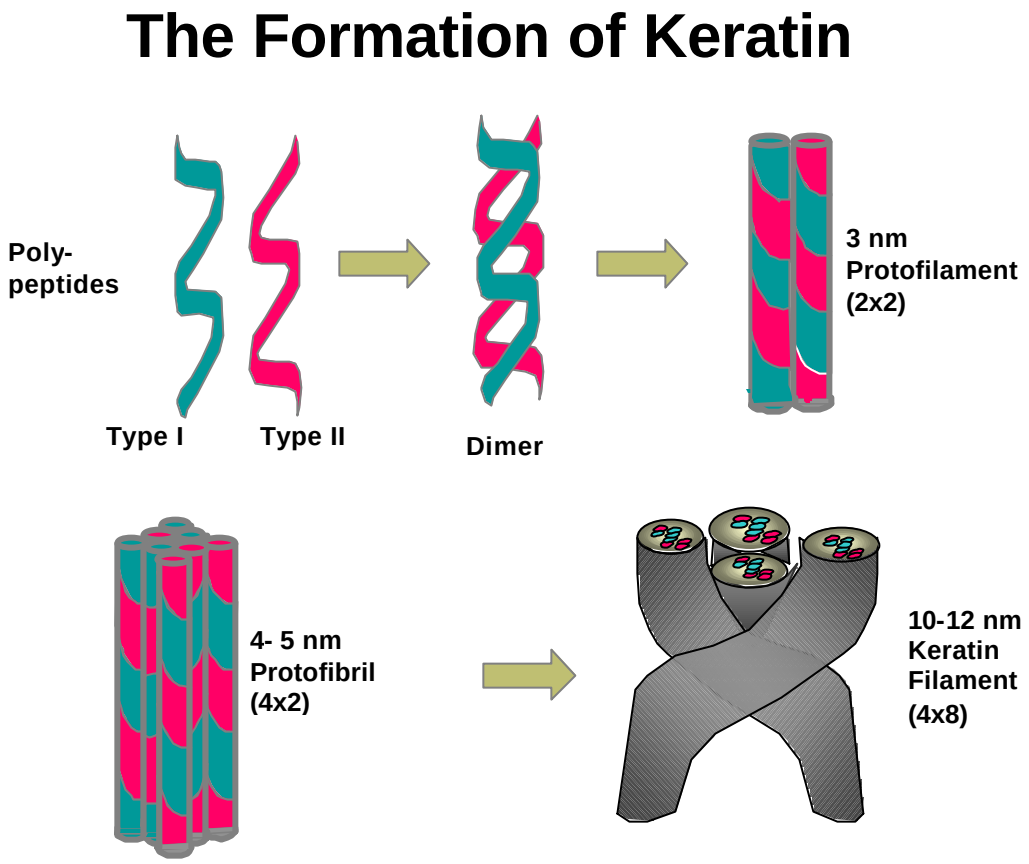

Figure 2. Organization of a keratin intermediate filament.

Most easily described as a pair of protofilaments staggered axially in such a way that the A_{12} mode stabilizes the filament. These protofibrils then pair laterally to form a 4.5 nm molecule. Finally, four protofibrils coil around each other in a left-handed manner to generate an intact KIF that consists of 32 monomer chains in cross-section (Steven *et al,*

1983; Parry and Steinert, 1999;) (Fig 2). The exact three-dimensional structure of the KIFs has not been elucidated, but recent studies have defined the crystal structure of the 2B domain of other intermediate filaments (Hermann and Aebi, 2000; Strelkov et al, 2002).

Keratin genodermatoses

Because keratins serve as the major cytoskeletal proteins of the epidermis, when the synthesis of a keratin protein is defective, the structural integrity of the affected keratinocytes and the stratum corneum they produce is affected (Reichelt *et al*, 1999; Schmuth *et al*, 2001). Over the past decade, morphologic and molecular analyses have shown that most cutaneous keratinization disorders in humans display a unique morphologic feature: epidermolysis. Epidermolysis is a form of keratinocyte necrosis that appears by light microscopy as cytoplasmic vacuolation and degeneration. Epidermolysis occurring within the granular cell layer is typically accompanied by large keratohyaline granules. Ultrastructurally, the characteristic features are clumping of keratin filaments and cytoplasmic vacuolation (Nazarro *et al*, 1990; Virtanen *et al*, 2001). These alterations within the superficial epidermis, accompanied by orthokeratosis, are characteristic features of a group of heritable diseases in humans which contain defects in the synthesis of one or more of the superficial keratins (Ackerman, 1970). Table 1 summarizes the cutaneous human diseases to date that are caused by heritable keratin mutations involving the epidermis.

The vast majority of keratin mutations are autosomal dominant and act by dominant negative interference at the protein level. The production of even a small amount of abnormal keratin polymerizing with normal keratin can disrupt the intermediate filament network and lead to tonofilament aggregation, cytoskeletal instability and epidermolysis.(Ishida-Yamamoto *et al*, 1992; Smith, 2003).

The first keratin gene mutation was documented in patients affected with the Dowling-Meara form of epidermolysis bullosa simplex (EBS) (Coulombe *et al*, 1991a; Coulombe *et al*, 1991b; Lane *et al*, 1992). The mutations in this disease resulted in amino acid substitutions in the helix initiation or helix termination motifs of keratin 5 (K5) or keratin 14 (K14) which caused epidermolysis in the basal layer. Other milder forms of EBS, Weber-Cockayne, Koebner and EBS with mottled pigmentation have subsequently been defined (Dunnill, 1998; Corden and McLean, 1996). EBS is largely an autosomal dominant disease, but occasional autosomal recessive mutations have been described (Batta *et al*, 2000; Corden and McLean, 1996)

Two generalized keratin genodermatoses affecting the suprabasal layers have been well-characterized: epidermolytic hyperkeratosis (EHK) and ichthyosis bullosa of Siemens (IBS). The molecular basis of EHK (also referred to as bullous congenital ichthyosiform erythroderma) was first attributed to genetic mutations in K1/K10 by Cheng et al in 1992. Although "hot spot" regions of these genes have been identified and most mutations occur in the 1A helix initiation motif, the 2B helix termination

Table 1. Cutaneous diseases in humans caused by keratin gene mutations.

Keratin	Disease	Reference
Keratin 1	Epidermolytic hyperkeratosis Diffuse non-epidermolytic palmoplantar hyperkeratosis Epidermolytic palmoplantar keratoderma Striate palmoplantar keratoderma Ichthyosis hystrix of Curth-Macklin	Chipev *et al*, 1992 Kimonis *et al*, 1994 Terron-Kwiatkowski *et al*, 2002 Whittock *et al*, 2002 Sprecher *et al*, 2001
Keratin 2e	Ichthyosis bullosa of Siemens	McLean *et al*, 1994b
Keratin 4	White sponge nevus	Rugg *et al*, 1995
Keratin 5	Epidermolysis bullosa simplex	Lane *et al*, 1992
Keratin 6a	Pachyonychia congenita 1 Pachyonychia congenita 2	Bowden *et al*, 1995 Ward *et al*, 2003
Keratin 6b	Pachyonychia congenita 2	Smith *et al*, 1998
Keratin 9	Epidermolytic palmoplantar keratoderma	Reis *et al*, 1994; Torchard *et al*, 1994
Keratin 10	Epidermolytic hyperkeratosis Annular epidermolytic ichthyosis	Cheng *et al*, 1992 Joh *et al*, 1997
Keratin 13	White sponge nevus	Richard *et al*, 1995
Keratin 14	Epidermolysis bullosa simplex	Bonifas *et al*, 1991; Coulombe *et al*, 1991a; Coulombe *et al*, 1991b
Keratin 16	Pachyonychia congenita 1 Focal non-epidermolytic palmoplantar hyperkeratosis Unilateral palmoplantar verrucous nevus	McLean *et al*, 1995 Shamsher *et al*, 1995 Terrinoni *et al*, 2000
Keratin 17	Pachyonychia congenita 2 Pachyonychia tarda Steatocystoma multiplex	McLean *et al*, 1995; Smith *et al*, 1997 Paller *et al*, 1991 Smith *et al*, 1997

motif or the H1 domain, mutations have occurred at many other sites in the keratin molecule (Compton, 1994; Virtanen *et al*, 2003). Currently, 47 mutations leading to EHK, 20 in *KRT1* and 19 in *KRT10* have been documented in the literature (McLean *et al*, 1999). The majority of these mutations are single heterozygous point mutations, although a few dinucleotide substitutions and one complex deletion/insertion have been described (Joh *et al*, 1997; McLean *et al*, 1999; Virtanen *et al*, 2003). All cases reported to date are autosomal dominant.

IBS is a scaling disorder that clinically and histologically can be difficult to distinguish from mild forms of EHK (Irvine and McLean, 1999). In people, the absence of erythroderma and the molting of the outer layers of the epidermis resulting in more scaling than erosion (the 'Mauserung phenomenon') are used to distinguish this disease from EHK clinically (Irvine *et al*, 2000). The disease is caused by mutations in K2e that occur predominantly in the helix termination motif (Smith, 2003). IBS is a rare disease, and all mutations reported to date are autosomal dominant (Whittock *et al*, 2001).

Epidermolytic palmoplantar keratoderma (EPPK) is an epidermolytic hyperkeratotic disorder confined to the palmar and plantar epidermis. This disease is typically due to a mutation in keratin 9 (K9) that is only expressed in palmar and plantar epidermis (Langbein *et al*, 1993). The majority of the K9 mutations associated with EPPK have occurred in the helix initiation motif (Reis *et al*, 1994) with a single report of a mutation occurring outside of this region in K9 (Coleman *et al*, 1999). As would be expected because K1 is the type II heterodimer for K9, rare mutations in K1 have accounted for a mild form of EPPK (Hatsell *et al*, 2001). In addition, mutations in the

V1 domain of K1 have been the cause of diffuse non-epidermolytic palmoplantar keratoderma (NEPPK).

We have recently identified a heritable disorder in Norfolk Terriers that has all of the morphologic hallmarks of a keratinization disorder. In these dogs, lesions are first noted shortly after birth when mild trauma results in superficial intraepidermal blister formation. As the dogs age, blisters are less conspicuous and hyperpigmentation and scaling, especially in frictional areas, are the major clinical findings. Histologically there is moderate epidermal hyperplasia and epidermolysis occurs linearly within the upper granular layer. The stratum corneum is orthokeratotic with a thin, wispy morphology. Electron microscopic examination of two dogs has shown diminution of keratin filaments in the upper epidermis replaced by aggregates of clumped keratin and marked cytoplasmic vacuolation. In addition, prominent and often abnormally shaped keratohyaline granules are frequently observed, and the number of lamellar bodies is increased. A major difference noted between superficial keratinization defects in humans and Norfolk terriers is that the canine disease appears to be recessive; whereas, as previously stated almost all keratinization disorders in humans are dominant (Chu and Weiss, 2002).

Identification of a keratin 10 mutation in Norfolk terrier dogs

A complete genomic sequence of canine KRT10 (GenBank accession AY318944 under submission) was determined using oligonucleotide primers for PCR designed initially from homologous regions of previously reported human and mouse DNA

sequences and later from canine sequence. DNA sequencing of a PCR product resulting from amplification of genomic DNA from affected dogs using a canine specific forward primer in exon 4 (5'TCTAGCAGGCTGCGGTAGG3') paired with a reverse primer in exon 6 (5'GATGCTGAAGCCTGGTTCAATG3') revealed a single base GT to TT change in the first nucleotide of the consensus donor splice site of intron 5 (Fig 3). The donor splice site is essential for directing the proper intron splicing. The consensus sequence is GTA/GAGT. Some variability within this sequence may be tolerated, but the initial GT is considered almost invariant (Strachan and Read, 1999).

Sequencing of the same amplification product in DNA samples from obligate heterozygotes showed that both the wild type allele and TT mutant allele were present (Fig 3). The expected GT consensus sequence was present in multiple normal Norfolk terrier dogs not from lines related to affected dogs and in multiple other breeds. No additional nucleotide differences between normal and affected dogs were identified.

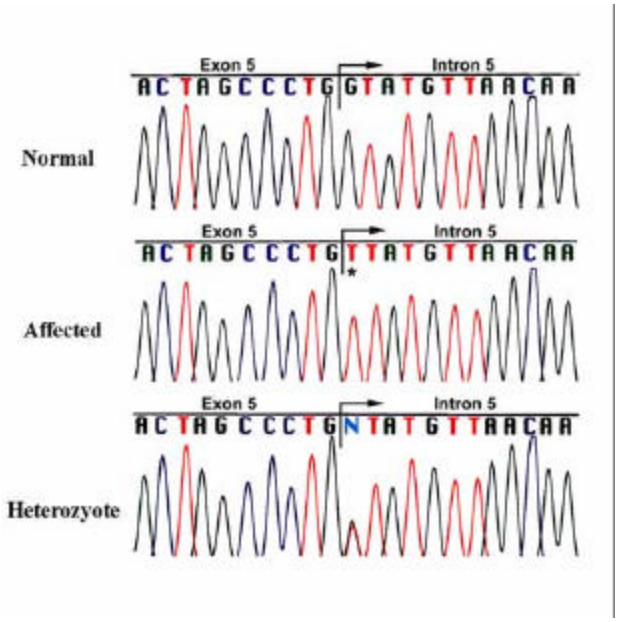

Figure 3. Chromatograms from genomic DNA of normal, heterozygote and affected dogs. Affected dog DNA has a G? T switch in the initial 5' nucleotide of intron 5. Heterozygote DNA does not have a consensus nucleotide at the same position indicating a G and T mixture.

To examine the effects of the splice site mutation, the primer set that identified the mutation in the genomic DNA was used to amplify canine KRT10 cDNA from affected and homozygous wild type Norfolk terrier dogs. Agarose gel electrophoresis revealed an expected single 359bp band in normal dogs. In affected dogs, the 359bp band was not detected, but a prominent 330 bp band and multiple smaller and larger bands were identified (Fig 4). Three of these amplification products were isolated, re-amplified and sequenced directly. The smallest band (approximately 230bp) represented the in-frame and entire removal of exon 5 (126 nucleotides) and could be explained by the alternative use of the normal intron 4 GT site. If translated, this mis-spliced transcript would produce a deletion of 42 amino acids from the proximal portion of the 2B rod domain

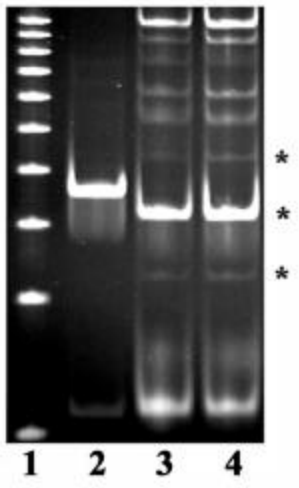

Figure 4. Agarose gel electrophoresis of cDNA from normal and affected Norfolk terrier dogs using primers that span exon 5 and intron 5. Lane 2 contains normal cDNA and shows a single 359bp product. Lanes 3 and 4 contain affected cDNA and show multiple smaller and large bands. (*) denotes the three bands that were subsequently sequenced.

Before the stutter (Fig 5). The brightest amplification product (approximately 330bp) corresponded to a 35bp out-of-frame deletion of the end of exon 5. This likely occurred due to activation of a cryptic splice site within exon 5 (GTCTG). The sequence of this product was out of frame and contained an insertion of 114 incorrect nucleotides followed by a premature termination codon. If translated, the mis-spliced transcript would encode for 38 incorrect amino acids in the mid 2B domain and then termination of the molecule (Fig 6). Sequencing of a third larger band (approximately 450bp) revealed a 95bp out-of-frame inclusion at the 5' end of intron 5 that likely resulted from the alternative use of a GT sequence within intron 5 (GTAAG) which created a premature termination codon after insertion of six incorrect nucleotides at the end of exon 5. The result of this premature termination was truncation of the molecule in the mid 2B region (Fig 7).

If translated, the abnormal transcripts from these three cryptic/alternative splice sites would result in either a keratin molecule with a loss of a portion of the 2B domain or an incomplete protein that terminates within the mid 2B rod domain 3' to the region (Yasukawa *et al*, 2002). The remaining bands (PCR amplification products) in Figure 4 were not examined and may represent other mis-spliced transcripts or products generated from small amounts of genomic DNA contaminating the cDNA samples.

In all affected dogs, the phenotype correlated with a homozygous mutation in the GT consensus sequence of the donor splice site in intron 5 of KRT10. This represents the first time a spontaneous keratin mutation has been confirmed in a dog or any mammalian species other than human.

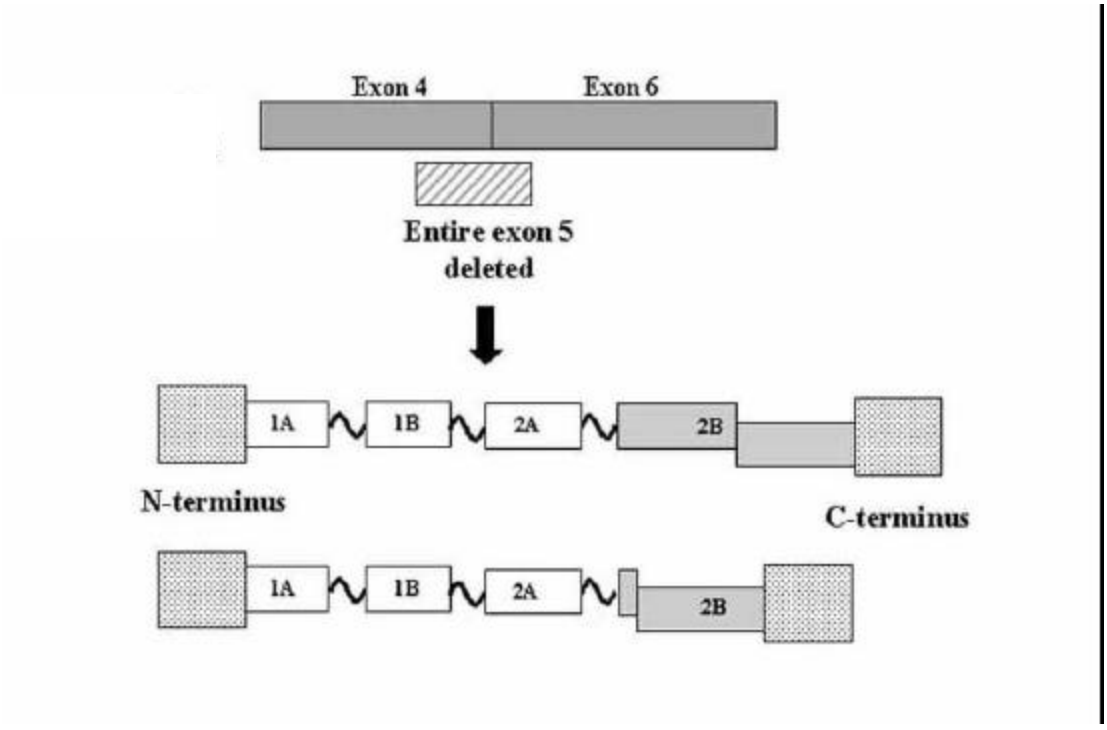

Figure 5. In-frame removal of complete exon 5.

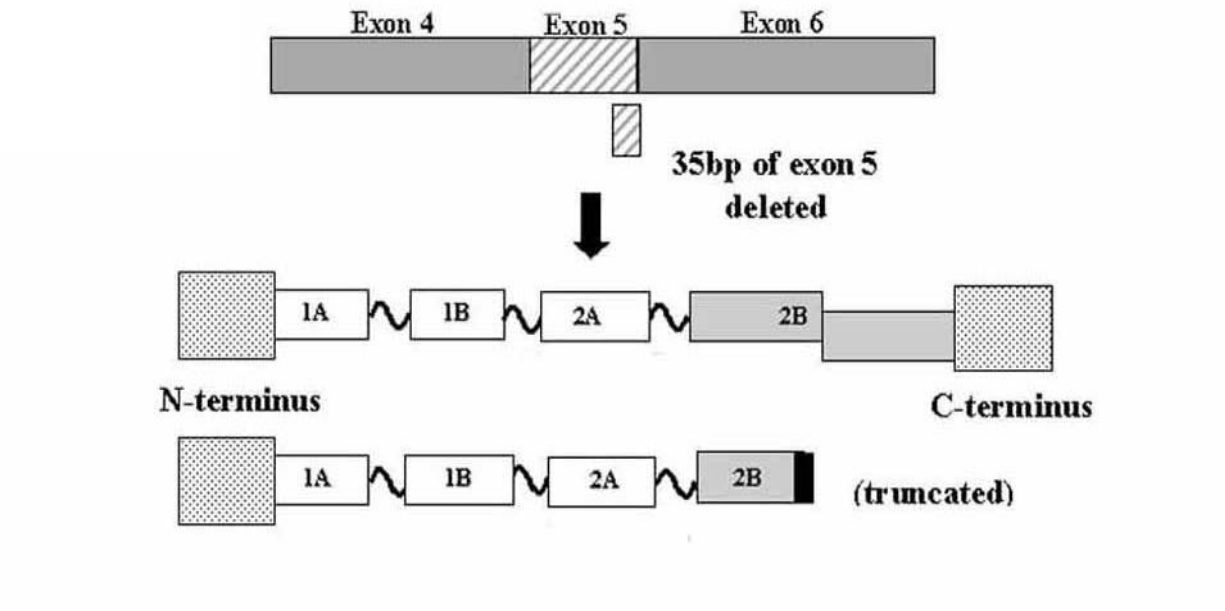

Figure 6. Partial deletion of the 3' end of exon 5

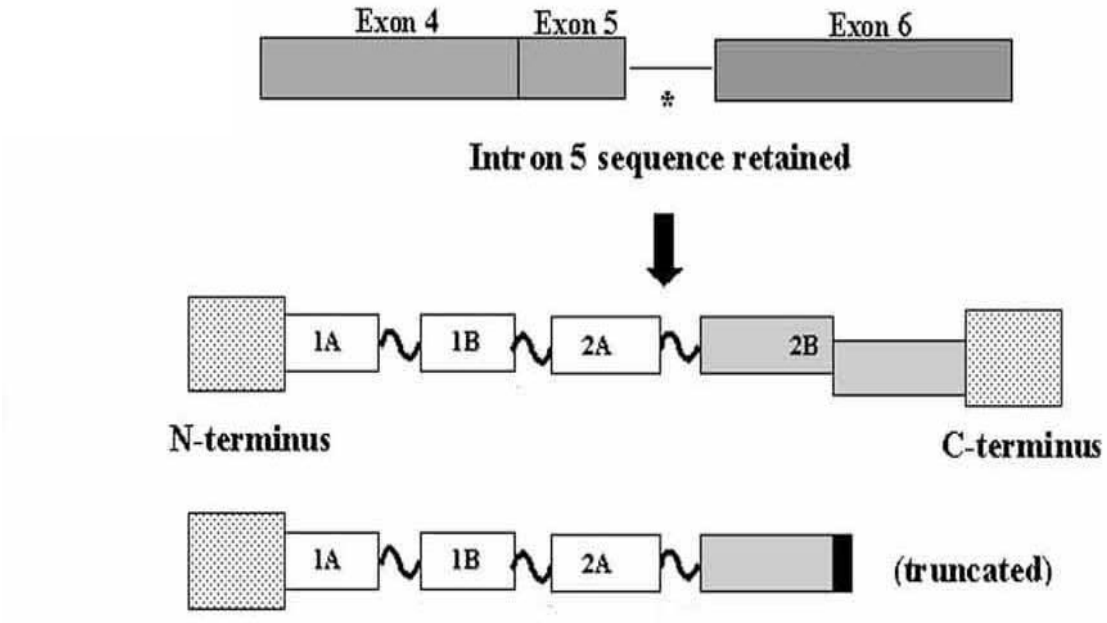

Figure 7. Partial retention of intron 5

The mild phenotype correlates with other mutations in the helical region of the 2B domain upstream from the conserved helix termination motif. In humans, mutations in the same region of KRT10 or KRT1 have been associated with mild EHK, mild epidermolytic PPK and mild combined EHK/PPK. (Syder *et al*, 1994; Hatsell *et a*l, 2001; Terron-Kwiatkowski *et al*, 2002).

As with other structural proteins that form multimers, mutations affecting keratins usually have a dominant negative effect**,** and recessive forms of these diseases are less commonly recognized (Smith, 2003). The majority of recessive keratin disorders are forms of epidermolysis bullosa simplex caused by mutations in KRT14 (Ciubotaru *et al*, 2003; Lanschuetzer *et al*, 2003; Smith 2003), with a single report in KRT5 (Yasukawa *et al*, 2002). Most of these mutations are nonsense mutations or missense mutations that result in premature termination codons early in the keratin molecule. To our knowledge, the disease in Norfolk terriers is the first confirmed recessive mutation of a suprabasal keratin.

Because of the shorter generational times and selective breeding practices of dogs, an unusual aspect of the Norfolk EHK was the ability to examine an eight generation pedigree that contained seven affected dogs and was consistent with an autosomal recessive mode of inheritance for the disease. Although some dogs are now deceased, we were able to confirm the genotypes of most of the 33 obligate heterozygotes (all clinically normal) with a mutation detection assay. Of the recognized hereditary disorders of the pure-bred dog, approximately 70% are recessive, a percentage higher than in humans (Patterson, 2000). The major factors that contribute to this higher

level of recessive diseases are first, the breeding practice of isolating specific lines of dogs in order to select for particular desirable traits. This practice can also result in more frequent matings of undetected carriers. Second, the gene pool available to individual breeds has become progressively smaller over decades of selection through a type of founder effect known as the popular sire effect. Third, recessive diseases may also predominate simply because dominantly transmitted diseases are easier to select against in dogs without the presence of a carrier state. Recognition of this disease suggests that recessive suprabasal keratin mutations may occur in humans but remain silent due to the rarity of consanguinity in human pedigrees.

The Norfolk terrier KRT10 mutation is associated with the use of multiple exonic and intronic cryptic donor sites and the normal intron 4 donor splice site sequence as alternative splice sites. When the cryptic donor sites are used, premature termination codons occur in the transcripts. When the intron 4 donor splice site is used, the nucleotide sequence remains in-frame, but the deletion of exon 5 results in a predicted loss of 42 amino acids in the first half of the 2B domain before the stutter. In affected dogs, we speculate that lesions result from the combination of markedly decreased filament synthesis, supported by the electron microscopy and immunoblot results, and the production of a small amount of mutated K10 derived from abnormally spliced in-frame transcripts, supported by the presence of abnormal filament aggregates ultrastructurally. Although we could amplify portions of KRT10-derived cDNA from the skin of affected dogs by PCR, the KRT10 mRNA levels were markedly lower than in breed-matched dogs homozygous for the wild type allele, suggesting that the presence of

premature termination codons or nucleotide alterations leads to nonsense mediated decay of KRT10 transcripts. If abnormal K10 synthesis does occur, our data indicate at least one mutated form would be altered in the central area of the 2B region, not within the helix termination motif.

In this study, examination of confirmed heterozygotes shows that these dogs were normal clinically, histologically and in terms of K10 protein expression; therefore, it appears that haploinsufficiency did not affect keratin intermediate filament assembly. Additionally, if abnormal K10 protein was produced, it did not disrupt the cytoskeleton sufficiently to produce epidermolysis.

Finally, this represents the first report of a keratin mutation in the intron 5 donor splice site. Splice site mutations have been reported rarely as causes of EHK and EPPK, due to mutations in KRT1, and as causes of EBS, due to mutations in both KRT5 and 14. The splice site mutation most similar to this case occurred in the donor site of intron 6 in KRT1 and led to a mild EPPK with rare hyperkeratotic lesions in other sites (Hatsell *et al*, 2001).

Since this mutation is outside of the "hot spot" regions for KRT10, it can provide more data in the on-going effort to catalog the mutations causing keratinization disorders and to define genotype-phenotype correlations. Further study of this disease in a novel species may provide insight into the function of keratin intermediate filaments and the pathogenesis of disorders of keratinization.

Specific objectives

There were four objectives of this project: 1) to define the phenotype of the Norfolk terrier keratinization disorder and utilize immunohistochemistry to compare the type and morphologic pattern of cytokeratin protein expression in normal skin and skin from affected Norfolk terriers, 2) to develop organotypic cell culture techniques that allow both normal and affected keratinocytes to cornify in culture, 3) to evaluate four specific genes involved with terminal differentiation (K1, K2e, K10 and TGM) for altered expression through RT-PCR and quantitative PCR of RNA obtained from both organotypic cultures and tissue biopsy samples of normal and affected Norfolk terriers, and 4) to use a commercially available cDNA microarray to globally determine which genes are differentially up-regulated between keratinocytes obtained from biopsy samples of both normal and affected Norfolk terriers.

The phenotype was characterized based on clinical signs, lesion distribution, and morphologic abnormalities as determined by histologic, immunohistochemical, and ultrastructural evaluation. Specifically, immunohistochemical staining for keratin 1 and keratin 10 was performed.

To accomplish the second objective, it was necessary to establish primary keratinocyte and fibroblast cell cultures from tissue biopsies obtained from normal and affected Norfolk terriers. Immunohistochemistry for keratin 1 and 10 was performed on cultured epidermis, and cells from these cultures provided protein and nucleic acids for the other objectives involving immunohistochemistry, microarray analysis and quantitative PCR.

Additional genes that are associated with cornification but require a higher degree of specificity than provided for by cross-hybridization on cDNA microarray (specifically, keratin 1, keratin 2e, keratin 10 and transglutaminase 1), were quantitated with TaqMan RT-PCR. Primers were designed for each keratin that avoided the H1 and H2 homologous regions of the keratin genes as well as the trigger motifs. Expression levels of these 4 genes were evaluated from total RNA extracted from both normal and affected epidermis and normal and affected keratinocytes obtained from organotypic cell culture.

Microarray analysis was performed using a commercially available human microarray constructed from genes with known expression in epidermal keratinocytes or dermal fibroblasts (DermArray, Integriderm Birmingham, AL). Cross-hybridization techniques were successfully established, and a list of differentially expressed genes between normal, heterozygous and affected Norfolk terrier dogs was established. Validation of the microarray was accomplished with both SYBR green and TaqMan quantitative (real time) RT-PCR.

Summary

In summary, the overall objectives of this project were twofold: 1) to establish the techniques of cell culture, immunohistochemistry, cDNA microarray, T7 RNA amplification and quantitative (real time) PCR to define the process of cornification in the dog in both *in vitro* and *in vivo* systems, and 2) to apply these techniques to compare and contrast the biochemical process of cornification in normal dogs and dogs with a heritable defect in keratin synthesis.

CHAPTER II

A HERITABLE KERATINIZATION DEFECT OF THE SUPERFICIAL EPIDERMIS IN NORFOLK TERRIERS*

Overview

Although well-characterized in humans, abnormal cornification secondary to heritable superficial keratin defects is rarely reported in animals. This report describes the histological, ultrastructural and immunohistochemical features of a mild cornification defect in seven related Norfolk terrier dogs. Lesions were present at birth and pedigree analysis suggested an autosomal recessive mode of inheritance. The affected dogs had hyperpigmented skin with scaling following mild trauma. The lesions were generalized but most prominent in the glabrous skin of the axillary and inguinal regions – areas where the epidermis is not protected by hair and sustains frequent trauma. The most striking histological change was vacuolation in the upper epidermis, which often resulted in epidermolysis and blister formation. All of the affected dogs showed similar gross and histological changes. Ultrastructural changes included abnormal keratin filament clumping, prominent clear spaces in the cytoplasm of suprabasal keratinocytes, and abnormal keratohyaline granules. Immunohistochemical labeling for keratin 10 demonstrated a lack of expression in the superficial epidermis of affected dogs. All of the morphological changes noted in the Norfolk terriers were

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consistent with a mild form of a heritable defect in superficial keratin synthesis.

Introduction

Cornification is the end-product of epidermal differentiation that results in the formation of the outermost layer of the skin, the stratum corneum. It is a complex process that requires the carefully orchestrated convergence of three biochemical pathways, namely (1) formation and organization of keratin intermediate filaments, (2) formation and dispersion of intercellular lipids and (3) synthesis of the cornified envelope (Smack *et al*, 1994; Blumenberg and Tomic-Canic, 1997). These components of the stratum corneum form a life-sustaining barrier that protects the body against external, chemical, microbial and ultraviolet injury and prevents the loss of body fluids. (Credille *et al*, 2001; Kalinin *et al*, 2002). Once keratinocytes fully cornify, they are called corneocytes. New corneocytes that form in the lower regions of the stratum corneum move toward the surface and are sloughed in the process of desquamation (Piérard *et al*, 2000; Credille, *et al*, 2001).

Keratins are among the most abundant proteins found in epithelial cells, and their primary function is to create scaffolding that provides structural support and protects the cells from both mechanical and non-mechanical stresses (Irvine and McLean, 1999; Coulombe and Omary, 2002). The formation of the keratin intermediate filament network is a tightly-regulated event in which keratinocytes express different keratins as they migrate through the epidermal layers. Keratins exist as obligate heterodimers between a type I (acidic) and type II (basic) keratin (Steinert, 1993). Under normal

growth conditions, cells in the basal layer express keratins 5 (K5) and 14 (K14) while keratinocytes in the suprabasal layer express keratins 1 (K1) and 10 (K10). An additional keratin, K2e, is identified in the upper suprabasal layers of the epidermis and is considered a marker for late differentiation. (Smith *et al*, 1999; Bloor *et al*, 2003) The dimerization partner for K2e is not currently known. Appropriate synthesis of these superficial keratin pairs is essential for effective terminal differentiation of keratinocytes and structural stability of the epidermis (Fuchs *et al*, 1994).

Many heritable keratin defects leading to abnormal cornification of the stratum corneum have been identified in people. The sites of keratin expression determine the location of the disease in the epidermis; however, the phenotype varies with the location of the mutation in the gene and its effects on both individual keratin synthesis and heterodimer formation (Irvine and McLean, 1999; Ishida-Yamamoto and Takahashi, 2002). In almost all heritable keratin defects, the clinical disease is associated with epidermal fragility, a change that appears histologically as epidermolysis (Smith *et al*, 1999). Mutations in the basal keratins K5 and K14, a disease termed epidermolysis bullosa simplex, result in basalar epidermolysis (Olivry and Jackson, 2001; Yasukawa *et al*, 2002). Mutations in the superficial keratins, K1/K10 and K2e, result in upper spinous or intragranular epidermolysis. Epithelialization that adequately compensates for epidermolysis, results in the disease epidermolytic hyperkeratosis (EHK) (Syder *et al*, 1994; Arin *et al*, 1999). When epidermolysis predominates over hyperkeratosis, sloughing and vesicles are the major clinical features. This phenotype is seen in ichthyosis bullosa of Siemens (IBS), a disease that results from defects in K2e synthesis

(Kremer *et al*, 1994; McLean *et al*, 1994b). Keratin 9 (K9) is a superficial keratin that pairs with K1 but whose expression is restricted to palmoplantar epidermis. Mutations in K1 and K9 have also been associated with a regional variant of EHK termed epidermolytic palmoplantar hyperkeratosis (EPPHK) (Hatsell *et al*, 2001). Reports of keratin defects are rare in the veterinary literature, but spontaneous occurrences of EHK in a Labrador retriever (Mecklenberg *et al*, 2000) and a mixed breed dog (August *et al*, 1988) have been described.

The present report describes a mild, heritable cornification defect resulting in excess scaling and hyperpigmentation. Histological, ultrastructural and immunohistochemical analyses of the cutaneous lesions revealed abnormal cornification with features characteristic for a heritable defect in superficial keratin synthesis.

Materials and methods

Samples and histopathology

Biopsy samples from affected skin of seven Norfolk terrier dogs were sent to the diagnostic dermatopathology services at Michigan State University and Texas A&M University for histological evaluation from 1996 to 2001. All tissues were fixed with 10% neutral buffered formalin and embedded in paraffin wax by routine methods. Skin sections (3 to 5 µm) were stained with haematoxylin and eosin (HE).

Immunohistochemistry

This was performed on formalin-fixed section with antibodies 34ßB4 (Enzo,

Farmingdale, NY, USA) and DE-K10 (Dako, Carpinteria, CA, USA), which labelled K1 and K10 respectively, and which had been shown to display the appropriate cross-reactivity with canine antigens (Mecklenberg *et al*, 2000). Sections (5 µm), placed on slides coated with 3-aminopropyl-triethoxysilane, were air-dried overnight, dewaxed and rehydrated. Antigen retrieval for K1 consisted of enzyme pre-treatment with trypsin at 25? C for 10 min. To improve antigen retrieval for K10, the sections were heated in a citrate buffer (Antigen Unmasking Solution; Vector Laboratories, Burlingame CA, USA) in a waterbath at 97? C for 20 minutes. All sections were treated with Universal Block (KPL, Gaithersburg MD, USA) to inhibit endogenous phosphatase activity. The primary antibodies were diluted with Common Antibody Diluent (Biogenex, San Ramon CA, USA) and visualized with a commercially available kit (Ultra Streptavidin Alkaline Phosphatase; Signet Pathology Systems, Dedham MA, USA) according to the manufacturer's instructions. HistoMark Red (KPL, Gaithersburg MD) served as the chromogen. Sections of normal canine epidermis served as a positive control. Antisera that did not react with the canine control tissue served as negative controls.

Transmission electron microscopy (TEM)

Tissues were post-fixed with osmium tetroxide 1% in 0.1M phosphate buffer, dehydrated through graded alcohol solutions, transferred to propylene oxide, infiltrated with Poly/Bed 812:Araldite:DSSA resin, 5:4:12 (Polysciences, Inc., Warrington, PA, USA) and cured at 60° C for 48 hrs. Sections were made with a diamond knife and stained with aqueous uranyl acetate and lead citrate. Micrographs were taken with a Phillips 301 Transmission Electron Microscope.

Results

Clinical history

Seven dogs showed a very similar disease progression and spectrum of clinical signs. Mild cutaneous abnormalities noted shortly after birth consisted of a grayish discoloration of the skin and excessive scaling on the ear margins. Mild forms of trauma (e.g. towel drying) produced shallow blisters, which were particularly severe in the inguinal and axillary regions (Fig 8). As the dogs aged, they developed marked hyperpigmentation (Fig 9A) and a mild to moderate degree of generalized scaling (Fig 9B). The dogs were usually malodorous and required frequent bathing to control the scaling as well as the secondary bacteria- and yeast-related dermatitis.

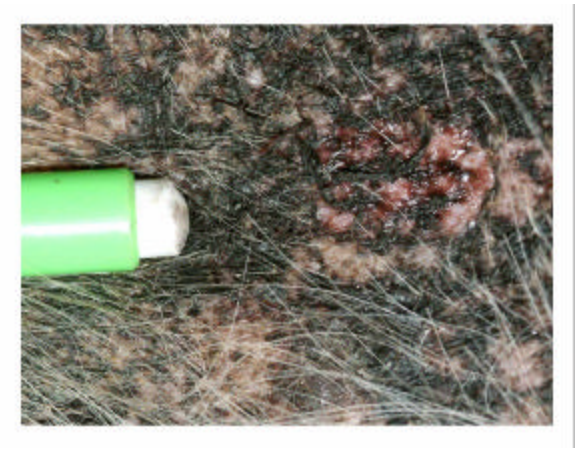


Figure 8. Adult affected Norfolk terrier dog. Erosion formation following mild trauma with a pencil eraser.

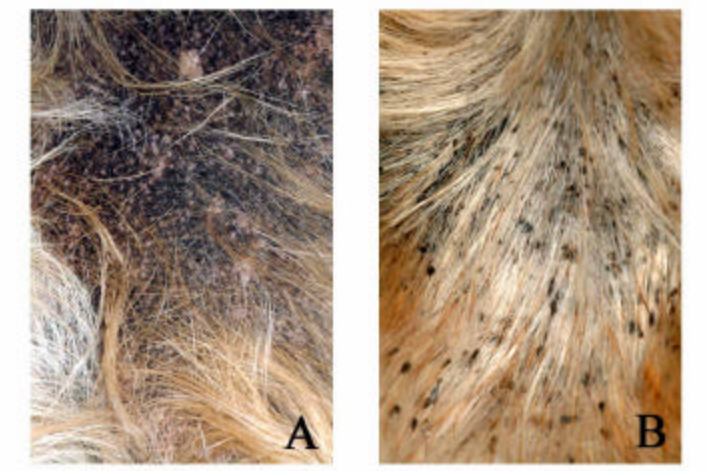


Figure 9. Clinical photos from an affected Norfolk terrier dog.
(A) Generalized hyperpigmentation, (B) Scaling

Histopathology

The histological changes in the epidermis correlated with the clinical features of the disease. The epidermis was moderately hyperplastic and hyperpigmented with orthokeratotic hyperkeratosis. The granular and spinous cell layers contained cytoplasmic vacuoles (epidermolysis) accompanied by intracytoplasmic eosinophilic inclusions consistent with keratin filament aggregates, and numerous variably sized and sometimes extremely large keratohyaline granules (Fig 10). The stratum corneum was composed of multiple layers of thin, wispy corneocytes (Fig 11). Areas of separation associated with cytolysis and subsequent clefting and vesicle formation occurred in the upper granular cell layer (Figs 10, 11). These changes were consistent with the superficial sloughing of the upper epidermis noted with gentle trauma (see above). The basal cell layer appeared normal.

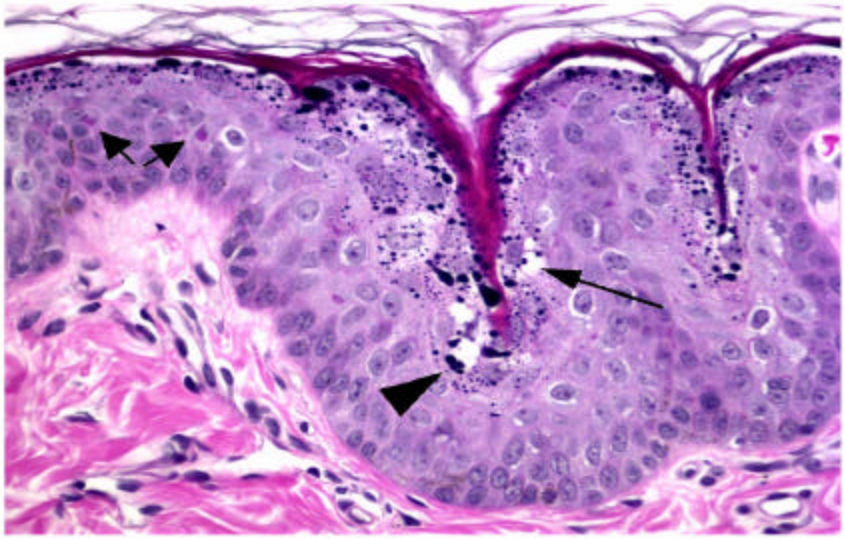


Figure 10. Epidermis from affected dogs. Suprabasal keratinocytes frequently contain eosinophilic cytoplasmic granules (short arrows) and display cytoplasmic vacuolation (long arrow). Keratohyaline granules are increased in number, irregularly shaped and frequently enlarged (arrowhead). HE. X 200.

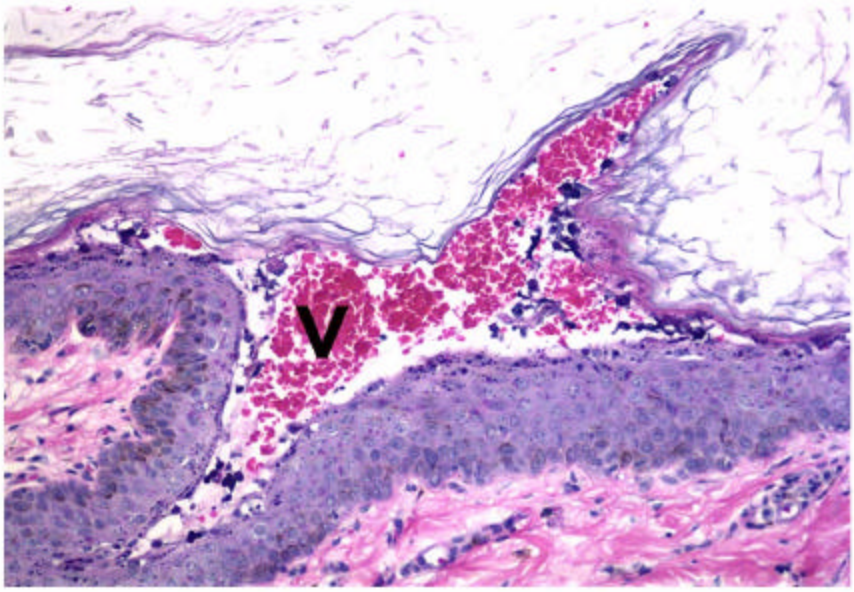


Figure 11. Vesicle formation in the granular cell layer. Epidermal hyperplasia and hyperkeratosis with intragranular vesicle formation (v). HE. X 200.

Immunohistochemistry

This confirmed that labelling for K1 and K10 was limited to the superficial layers of the epidermis in normal dogs. (Figs 12, 13) Positive labelling for K1 (Fig 14) but not K10 (Fig 15) was noted in the superficial epidermis of affected dogs.

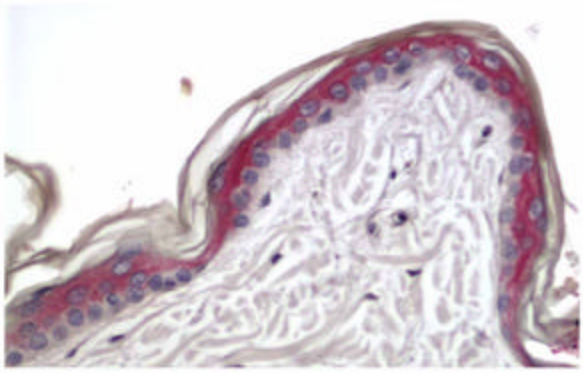


Figure 12. Immunohistochemical staining for cytokeratin 1 in normal dogs. Diffuse cytoplasmic staining is present uniformly throughout the suprabasal epidermal layers. Alkaline phosphatase, Mayer's haematoxylin counterstain. X 400.

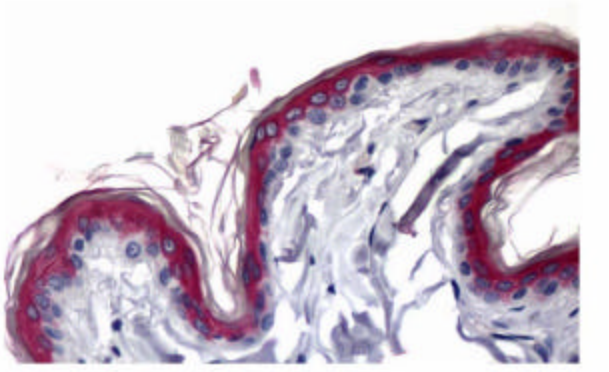


Figure 13. Immunohistochemical staining for cytokeratin 10 in normal dogs. Diffuse cytoplasmic labelling for cytokeratin 10 in the suprabasal keratinocytes of normal dogs. Alkaline phosphatase, Mayer's haematoxylin counterstain. X 400.

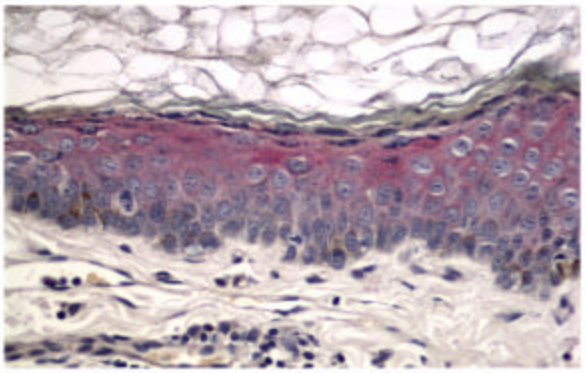


Figure 14. Immunohistochemical staining for cytokeratin 1 in affected dogs. Diffuse cytoplasmic staining is present uniformly throughout the suprabasal epidermal layers. Alkaline phosphatase, Mayer's haematoxylin counterstain. X 400.

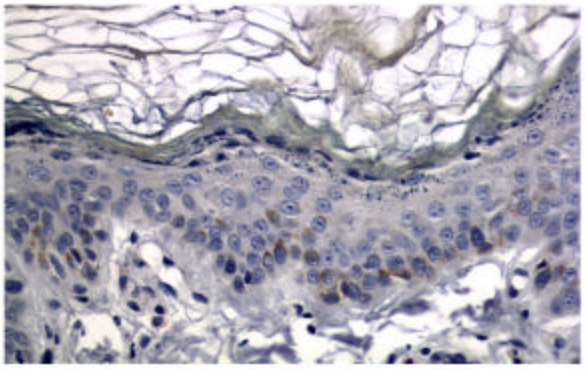


Figure 15. Immunohistochemical staining for cytokeratin 10 in affected dogs. Suprabasal keratinocytes show no evidence of cytoplasmic labelling for cytokeratin 10. Alkaline phosphatase, Mayer's haematoxylin counterstain. X 400.

TEM

Ultrastructural abnormalities indicating abnormal keratinization and subsequent epidermolysis were detected in the suprabasal layers while the basal cell layer appeared

normal. Loss of the normal keratin intermediate filament network was evident in both the spinous and granular cell layers. Variably sized, circular to irregular areas of cytoplasmic electron-lucency were often present (Figs 16, 17). These changes occurred most prominently in the granular cell layer where the keratin intermediate filament aggregates

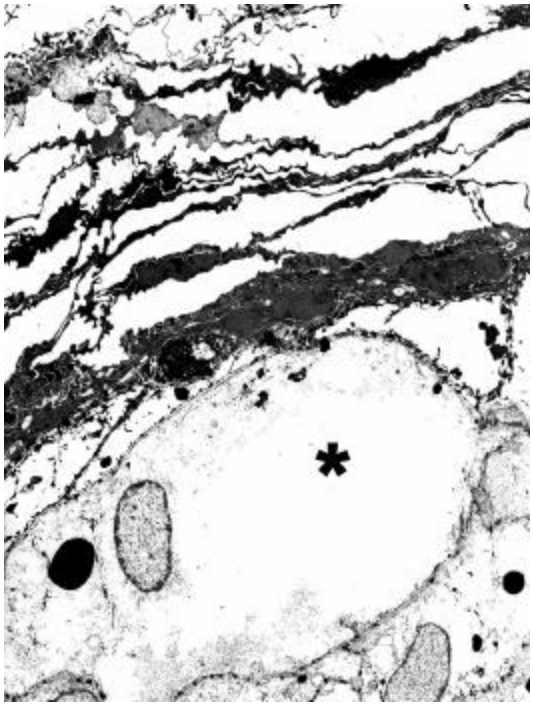


Figure 16. Upper suprabasal layers of affected epidermis. Large, cytoplasmic, electron-lucent spaces (*), considered secondary to disruption of the normal keratin intermediate filament network. TEM. X 4940.

Were frequently associated with enlarged and irregularly-shaped keratohyaline granules (Fig 18). In the upper suprabasal layers, large electron-lucent spaces often coalesced to form areas of separation and cell lysis, reflecting the vesicles seen clinically (Fig 17). An increase in the number of lamellar bodies was frequently noted in the granular cell layer

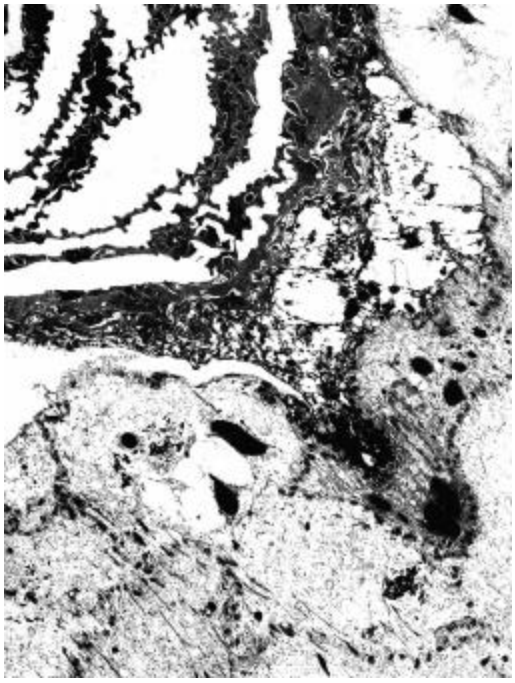


Figure 17. Upper suprabasal layers of affected epidermis.
Large electron-lucent spaces between multiple keratinocytes (arrows), reflecting the blisters seen clinically. TEM. X 6500.

(Fig 19). The stratum corneum consisted of thin, irregular corneocytes with highly convoluted and folded surfaces. These cells had a normal cornified envelope, but poorly formed keratin filaments made them appear collapsed (Figs 16, 17)

Genetic history

Pedigrees for the seven affected dogs were traced through seven generations and linked to a common ancestor (Fig 20). The pedigree data supported an autosomal recessive mode of inheritance by fulfilling several general inheritance characteristics: the tendency of the disease to skip a generation; the absence of affected parents producing affected offspring; and an average of approximately 25% affected dogs in a litter. In

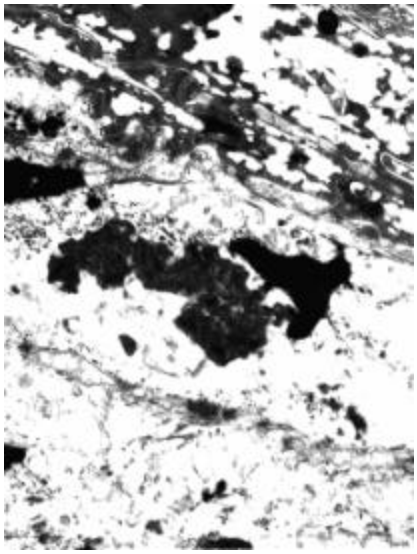


Figure 18. Granular cell layer of affected epidermis.
Irregularly shaped keratohyaline granule (arrow) associated with an abnormal aggregate of keratin filaments (arrowhead). TEM. X 6500.

Figure 19. Granular cell layer of affected epidermis.
Increased number of lamellar bodies (arrow). TEM. X 11 250.

Addition, males and females were affected with approximately equal frequency (males 57%, females 43%) making the possibility of a sex-linked trait unlikely (Oberbauer and Sampson, 2001). None of the affected dogs had affected mothers; consequently, a mitochondrial mode of inheritance was also excluded (Takahasi *et al*, 2002).

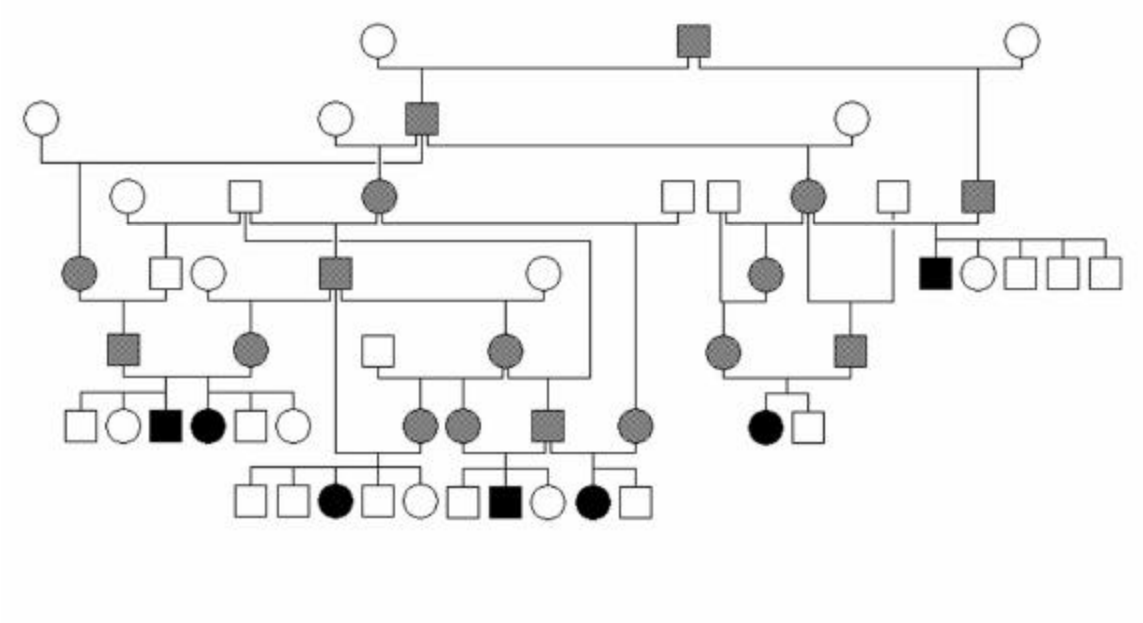


Figure 20. Pedigree of Norfolk terriers with a heritable keratinization defect.
Solid symbols represent affected dogs; shaded symbols represent unaffected obligate carriers; open symbols represent clinically normal dogs. Squares represent male dogs; circles represent female dogs.

Discussion

The clinical signs in all seven affected Norfolk terriers closely resembled those described for defects in K2e, K1 and K10 synthesis. Based solely on histological and

ultrastructural features, the canine disease has much more in common with IBS; however, immunohistochemical analysis suggested that the disease was most likely due to a defect in K10 synthesis.

Because K1 and K10 are obligate heterodimers, mutations in highly conserved regions of either the K1 gene (KRT1) or the K10 gene (KRT10) can produce a similar phenotype on non-palmar/plantar skin. Mutations in the K2e gene (KRT2e) usually cause a phenotype that is more blistering and less hyperkeratotic. EHK and IBS may be familial or occur sporadically, presumably due to a new mutational event. Once the mutation has occurred, both diseases follow an autosomal dominant mode of inheritance (Bale *et al*, 1993; DiGiovanna and Bale, 1994; Whittock *et al*, 2001).

In human patients, cutaneous lesions of all keratin defects are present at birth. Those suffering from K1/K10 defects typically present with widespread blisters and superficial erosions that result from keratin filament clumping and epidermolysis within the suprabasalar layers. Diffuse erythema is consistently noted at birth, and mild scaling may also be present (Cheng *et al*, 1992; Arin *et al*, 1999; Virtanen *et al*, 2001). With increasing age, the frequency of blistering decreases, and the skin becomes hyperkeratotic, particularly in the flexural areas (Bale *et al*, 1993). In the most severely affected patients, hard ridges of hyperkeratotic skin develop, which may cause contractures and decreased mobility. Hyperkeratotic skin is frequently colonized by infectious agents, and malodour is common.

Patients with K1/K10 defects have striking clinical heterogeneity, and studies correlating phenotypic variations with specific mutations. (Kremer *et al*, 1998; Michael

et al, 1999; Virtanen *et al*, 2001) suggest that the location of the mutation and the specific region of the keratin that is affected may be predictive of the degree to which an individual is affected (Terron-Kwiatkowski *et al*, 2002; Sybert *et al*, 1999). In addition, some specific phenotypic changes are dependent upon which of the two keratins is abnormal. Because K1 is the heterodimer for K9, KRT1 mutations are often associated with palmoplantar lesions, while such changes are generally absent in patients with mutations in KRT10 (DiGiovanna and Bale, 1994).

The main histological features of K1/K10 defects are hyperkeratosis, epidermolysis, keratin intermediate filament clumping, and vacuolization of the suprabasal keratinocytes (Sun *et al*, 2002). Abnormal keratin aggregates appear as eosinophilic cytoplasmic inclusions with HE staining. The degree of epidermolysis may be highly variable, with some patients displaying extensive cytolysis as deep as the first suprabasal layer and others showing cytolysis only in the upper spinous and granular cell layers (Cheng *et al*, 1992).

Ultrastructurally, the most prominent feature is the clumping of keratin intermediate filaments that begins in the first suprabasal layer of the epidermis. Aggregated keratins collapse in a shell-like network around the nucleus and create large clear spaces in the cytoplasm (Cheng *et al*; 1992; McLean *et al*, 1994a). Keratohyaline granules may be abnormally shaped and are often in close association with clumped keratin filaments (Ishida-Yamamoto *et al*, 1992; Anton-Lamprecht, 1994).

Human patients with mutations in the gene for K2e (KRT2e) typically have milder symptoms than those with mutations in KRT1 or KRT10. These patients are born

with generalized erythema and blistering that develops into dark-grey areas of hyperkeratosis, primarily on the arms, legs and flexural surfaces (Rothnagel *et al*, 1998). Distinct features used to distinguish IBS from EHK clinically are (1) the absence of erythroderma, (2) localization of hyperkeratosis to the flexural areas, and (3) presence of the "Mauserung phenomenon" (superficially denuded areas that occur at sites of previous blister formation and resemble molting) (Traupe *et al*, 1986). The histological and ultrastructural features are also very similar to those associated with K1/K10 defects but are confined to the upper spinous and granular cell layers of the epidermis (Basarab *et al*, 1999).

In the Norfolk terrier dogs, the heritable nature of the disease, the clinical evidence of epidermal fragility, and the presence of epidermolysis histologically suggested a defect in keratin synthesis. The mild clinical phenotype, absence of erythema and histological changes consisting of clefts in the upper granular zone suggested an IBS phenotype caused by a mutation in KRT2e. However, unlike the lesions in human IBS patients, those in the dogs were neither limited to, nor unusually severe in, the flexural areas. Unfortunately, we were unable to obtain a commercial antibody that cross-reacted with K2e in the dog, and immunohistochemical analysis could be completed only for K1 and K10. Based on the complete absence of staining for K10, a mutation in KRT10 was strongly suspected.

One notable difference between human EHK or IBS and the keratinization defect in the seven Norfolk terriers in this study was the mode of inheritance. To date, the majority of heritable keratin defects and all of the mutations associated with abnormal

K1, K10, or K2e synthesis follow an autosomal dominant mode of inheritance. The pedigree analysis of the Norfolk terriers; however, strongly supported an autosomal recessive mode of inheritance (Chu and Weiss, 2002).

Further studies are in progress to sequence KRT1, KRT10, and KRT2e in the dog and to identify a mutation that would explain the cornification defect present in the Norfolk terrier dogs described here.

CHAPTER III

PRESERVATION OF PHENOTYPE IN AN ORGANOTYPIC CELL CULTURE MODEL OF A RECESSIVE KERATINIZATION DEFECT OF NORFOLK TERRIER DOGS

Overview

Abstract: The purpose of this study was to reproduce *in vitro* the phenotype of a recessive keratinization defect of Norfolk terrier dogs characterized by a lack of keratin 10 (K10) production. Keratinocytes from skin biopsy samples of 4 normal and 2 affected dogs were cultivated under organotypic conditions at the air-liquid interface on collagen rafts with growth factor supplemented media to stimulate cornification. Cultured epidermis from normal dogs closely resembled normal epidermis *in vivo* and cornified. Cultured epidermis from affected dogs displayed many of the phenotypic alterations identified in skin biopsies. Immunohistochemistry and immunoblotting revealed a marked decrease in K10 from cultures of affected keratinocytes as compared to cultures of normal keratinocytes. Real-time RT-PCR quantitation showed a 31-fold decrease in keratin 10 (KRT10), a 1.75-fold increase in keratin 1 (KRT1) and a 136-fold increase in keratin 2e (KRT2e) between affected and normal epidermis. Organotypic keratinocytes showed a 241-fold decrease in KRT10, a 31-fold decrease in KRT1 and a 1467-fold decrease in KRT2e between affected and normal cultures. In summary, although keratin gene expression was altered *in vitro*, the morphology of normal and affected epidermis

was largely preserved. Thus, this culture system may provide an alternative to *in vivo* investigations for cutaneous research involving cornification in the dog.

Introduction

Keratins are polypeptides that polymerize to form keratin intermediate filaments, a major component of the cytoskeleton within epithelial cells (Smith *et al*, 2003). They are essential for complete cornification, the end-product of epidermal differentiation that results in the formation of the outermost layer of the skin, the stratum corneum. Cornification is a complex process that also requires concurrent formation and dispersion of intercellular lipids, and synthesis of the cornified envelope (Smack *et al*, 1994; Blumenberg and Tomic-Clonic, 1997). Once keratinocytes fully cornify, they are called corneocytes. New corneocytes that form in the lower regions of the stratum corneum move toward the surface and are sloughed in the process of desquamation (Piérard *et al*, 2000; Credille *et al*, 2001). Although their primary function is to create scaffolding that provides structural support and protects the cells from both mechanical and non-mechanical stresses, recent research has revealed a more dynamic range of function for keratins (Irvine and McLean, 1999; Coulombe and Omary, 2002). They are now recognized as having important roles in signal transduction, apoptosis and cell cycle activation (Paramio and Jorcano, 2002; Santos *et al*, 2002; Kirfel *et al*, 2003).

Currently, 24 epithelial keratins have been identified (Sprecher *et al*, 2002). They are separated into two groups based upon molecular weight and isoelectric point; acidic type I keratins (K9-K24) and neutral/basic type II keratins (K1-K8) that have a higher

molecular weight and are slightly longer than type I keratins (Coulombe and Omary, 2002). Keratin protein expression follows several general principles. They exist as obligate heterodimers and at least one member of each group must be coexpressed in all keratinocytes (Steinert, 1993; Weedon and Strutton, 2002). Keratins are developmentally regulated and not all are expressed during embryogenesis; furthermore, each tissue contains specific keratin pairs (Chu and Weiss, 2002).

Keratinocytes express different keratin pairs as they migrate through the epidermis. Under normal growth conditions, cells in the basal layer express keratins 5 (K5) and 14 (K14) while keratinocytes in the suprabasal layers express keratins 1 (K1) and 10 (K10) that are considered markers for terminal differentiation. An additional keratin, K2e, is identified in the upper suprabasal layers of the epidermis and is considered a marker for late differentiation (Smith *et al*, 1999; Bloor *et al*, 2003; Barnhart *et al*, 2004). During hyperproliferative conditions, three additional keratins (K6, K16, and K17) are expressed. Coordinated synthesis of these superficial keratin pairs is essential for effective terminal differentiation of keratinocytes and structural stability of the epidermis (Fuchs *et al*, 1994).

Many heritable keratin defects leading to abnormal cornification of the stratum corneum have been identified in humans (Weedon and Strutton, 2002). The location of the characteristic lesions in the epidermis is determined by the normal distribution of the keratin pair implicated in the disease. Regardless of which keratin pairs are affected, the clinical disease is associated with epidermal fragility, a change that appears histologically as epidermolysis (Smith *et al*, 1999; Ishida-Yamamoto *et al*, 2002). Mutations in K5 and

K14 result in basalar epidermolysis and a disease termed epidermolysis bullosa simplex (Olivry and Jackson, 2001; Yasukawa *et al*, 2002). Mutations in the superficial keratins, K1/K10 and K2e, result in upper spinous or intragranular epidermolysis. When epithelialization adequately compensates for the epidermolysis, the resulting disease is termed epidermolytic hyperkeratosis (EHK) (Syder *et al*, 1994). When epidermolysis predominates over hyperkeratosis, sloughing and vesicles are the major clinical features, and this phenotype is seen in ichthyosis bullosa of Siemens (IBS), a disease that results from defects in K2e synthesis (McLean *et al*, 1994b; Syder *et al*, 1994). Keratin 9 (K9) is a superficial keratin that pairs with K1, but its expression is restricted to palmoplantar epidermis. Mutations in K1 and K9 have also been associated with a regional variant of EHK termed epidermolytic palmoplantar hyperkeratosis (EPPHK) (Hatsell *et al*, 2001).

Although numerous keratin gene mutations have been identified in the human literature (Weedon and Strutton, 2002), currently no keratin mutations have been confirmed in domestic animal species, and reports of keratin defects in the veterinary literature are rare. Two reports have described the spontaneous occurrence of EHK in a Labrador retriever (Mecklenburg *et al*, 2000) and a congenital EHK-like lesion in a mixed breed dog (August *et al*, 1988).

An autosomal recessive cornification defect caused by a mutation in K10 (unpublished data) has been identified in 7 Norfolk terrier dogs with an extended pedigree. All of the affected dogs had similar clinical and histologic changes. Clinically, they demonstrated generalized hyperpigmentation and scaling with a tendency to develop erosions following mild trauma. Histologically, the most prominent feature was

vacuolation in the upper epidermis that resulted in epidermolysis and separation of the upper granular and spinous layer. Ultrastructural alterations included abnormal keratin filament clumping, prominent clear spaces in the cytoplasm of suprabasal keratinocytes and abnormal keratohyaline granules. Immunohistochemical staining for K10 was absent in the epidermis of affected dogs. The morphologic abnormalities identified in these dogs resembled a mild form of human EHK (Barnhart *et al*, 2004)

The goal of this study was to establish organotypic (OT) cell culture conditions that allowed the affected keratinocytes to be successfully cultivated and maintain the phenotypic alterations identified in affected skin. An *in vitro* model would provide a much larger and renewable source of keratinocytes for further studies involving protein and gene expression and diminish the need for multiple biopsies to investigate the disease.

Materials and methods

Sample acquisition

Six-millimeter punch biopsy samples were obtained from skin located in the dorsolateral thoracic region of 5 normal and 2 affected Norfolk terrier dogs. The age of the dogs ranged from 18 months to 9 ½ years. Biopsy samples were placed directly in chilled isolation media for immediate primary culture. Isolation media consisted of Williams Media E (WME) supplemented with gentamycin (20ug/ml), penicillin (2U/ml), streptomycin (2ug/ml) and amphotericin B (5ng/ml).

Submerged keratinocyte and fibroblast cultures

Keratinocytes were isolated from the biopsy samples and cultured by a modification of previously established conditions for long-term cultivation of canine keratinocytes (Wilkinson *et al*, 1987). Initial keratinocyte dissociation was accomplished with a 0.2% dispase solution(Invitrogen, Carlsbad, CA) at 37°C for 60-90 minutes. The keratinocytes were then propagated in WME supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, epidermal growth factor (10ng/ml), cholera toxin (10^{-10}M), gentamycin (20ug/ml), and 1X Antibiotic-Antimycotic (final concentration in media: 1U/ml penicillin, 1µg/ml streptomycin and 2.5ng/ml amphotericin B; Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator containing 5% CO_2. Fibroblasts were separated from the initial keratinocyte cultures by trypsinization (0.25% trypsin/1 mM EDTA) and propagated separately under identical conditions. The medium was changed every 2-3 days, and the cells were passaged at approximately 70% confluency by dissociating the monolayer with trypsin for 1-2 minutes at 37°C for fibroblasts and 8-10 minutes at 37°C for keratinocytes.

Fluorescence staining of submerged keratinocyte cultures

Keratinocytes from normal dogs grown under submerged culture conditions were harvested and stained with a pancytokeratin antibody (AE1 + AE3; MP Biomedicals, Irvine, CA), an antibody directed against K14 (LL002; Serotec, Raleigh, NC) and an antibody directed against K10 (RKSE60; Chemicon, Temecula, CA). Keratinocytes were removed by trypsinization (0.25% trypsin/1 mM EDTA) for 8-10 minutes at 37°C, pelleted and resuspended in fresh media. Coverslips were placed in 35 mm cell culture dishes, covered with 2ml of the media containing the keratinocytes and allowed to

incubate overnight at 37°C. After air drying, each coverslip was washed with phosphate buffered saline (PBS) and immersed in 4% paraformaldehyde for 5 minutes. The cells were then permeabilized by immersion for 5 minutes in a detergent solution containing 100uls nonidet P40 (Sigma-Aldrich, St. Louis, MO) and 100mls PBS and subsequently placed in humid chambers made with dampened filter paper and cell culture dishes. Approximately 50 µl of each primary antibody diluted 1:20 was pipetted onto a separate coverslip and allowed to incubate for 30 minutes at room temperature. After 3 rinses with PBS, approximately 50µl of the secondary antibody, FITC pan-IgG diluted 1:20, was pipetted onto each coverslip and allowed to incubate for 30 minutes at room temperature. The coverslips were then dried and mounted.

OT keratinocyte cell culture

Cultured keratinocytes from 5 normal and 2 affected dogs were seeded on both acellular collagen rafts and rafts containing fibroblasts cultured from the same dog. The collagen rafts were prepared with rat tail collagen type I (approximately 3-4 mg/ml) in 0.02N acetic acid (BD Biosciences, Bedford, MA). Eight volumes (12 ml) of chilled collagen were combined on ice with 1 volume (1.5 ml) of 10X Hank's Balanced Salt Solutionand 1 volume (1.5 ml) of 10% FBS and titrated to neutrality with 1M NaOH. To establish the cellular rafts, approximately 1.5 x 10^6 fibroblasts (final concentration 1 x 10^5 cells/ml) were added to the 1 volume of 10% FBS.

Subsequently, 2 ml of the collagen mixture was pipetted onto 6 cell culture inserts comprised of a transparent polyethylene terephthalate (PET) membrane containing 3 µm pores and suspended in a 6-well companion plate (BD Biosciences, Bedford, MA). The

collagen rafts were allowed to solidify for at least 4 hours at 37°C. Thin glass rings with a 21 mm inner diameter were placed on the rafts to compress the gel and allow uniform seeding of keratinocytes (Stark *et al*, 1999). The rafts were then allowed to equilibrate for 24 hours in WME containing the supplements previously listed for submerged cell culture plus transferrin (5µg/ml), hydrocortisone (0.4µg/ml), 2nM 3,3'-5 tri-iodothyronine and insulin (5µg/ml) (Jakic-Razumovic *et al*, 1994).

Keratinocytes derived from the third to fifth passages were grown to approximately 70% confluency, dissociated with 0.25% trypsin/1 mM EDTA and seeded on the collagen rafts at a density of approximately 1 x 10^6 cells/raft. The cells were then grown under submerged conditions by placing media directly on the raft and in the lower chamber. After 24-48 hours, the keratinocytes were approximately 60-80% confluent and the media was removed from the surface of the raft. Media was changed in the lower chamber every 2-3 days for 21 days.

Histopathology

Collagen rafts were harvested at days 7, 14 and 21, fixed with 10% neutral buffered formalin for 24 hours, trimmed into 2mm strips and subsequently embedded in paraffin wax by routine methods. All sections (5 to 7 µm) were stained with hematoxylin and eosin (HE).

Immunohistochemical staining

Immunohistochemistry for K10 was performed on formalin-fixed sections with RKSE60. Sections (5 µm) placed on slides coated with 3-aminopropyl-triethoxysilane were air-dried overnight, dewaxed and rehydrated. To improve antigen retrieval for K10,

the sections were heated in a citrate buffer (Antigen Unmasking Solution; Vector Laboratories, Burlingame CA) in a water bath at 97? C for 20 minutes. All sections were treated with Universal Block (KPL, Gaithersburg, MD) to inhibit endogenous phosphatase activity. The primary antibodies were diluted with Common Antibody Diluent (Biogenex, San Ramon, CA) and visualized with a commercially available kit, Ultra Streptavidin Alkaline Phosphatase (Signet Pathology Systems, Dedham, MA) according to the manufacturer's instructions. HistoMark Red (KPL, Gaithersburg, MD) served as the chromogen. Sections of normal canine epidermis served as a positive control, and antisera that did not react with the canine control tissue served as a negative control.

Immunoblotting

Keratinocytes were harvested from collagen rafts by solubilization in Laemmli-type sample buffer, and protein was quantitated using the BCA assay (Pierce, Rockford, IL). After quantitation, 4% ß-mercapto-ethanol was added to the extracts. Twelve micrograms of protein per sample were electrophoresed on a 10% tris-HCl/glycine/SDS polyacrylamide gel (BioRad, Hercules, CA) and proteins were transferred by overnight electroblotting to nitrocellulose membranes. The membranes were incubated with a 1:50 dilution of the primary K10 antibody, RKSE60. After washing in PBS, the membrane was incubated with a 1:2500 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). After washing, the phosphatase activity was detected with the chromogenic substrate BCIP/NBT (Sigma-Aldrich, St. Louis, MO).

RNA extraction and quantitation

Keratinocytes were removed from the collagen rafts by gentle scraping after 1 hour of trypsinization at 37°C and stored in RNALater (Ambion, Austin, TX) at –80°C. Epidermis from a skin biopsy sample of a normal dog was also obtained and stored at –80°C in RNALater. Total RNA was extracted with an RNAqueous-micro kit (Ambion, Austin, TX) and treated with DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. The RNA was quantitated with RiboGreen (Molecular Probes, Eugene, OR) and stored at –80°C.

Taqman quantitative real-time PCR analysis

Quantitative real-time RT-PCR was performed on total RNA obtained from epidermis of skin biopsy samples from 4 normal and 4 affected dogs as well as keratinocytes from 4 normal OT cultures and 2 affected OT cultures. Each reaction was performed in triplicate utilizing 50ng of total RNA/reaction and the TaqMan® One-step RT-PCR Master Mix reagents kit (Applied Biosystems, Foster City, CA). Gene-specific primers and a TaqMan® probe (containing an MGBNFQ (Minor groove binder/Non-fluorescent quencher) 3' label and a 6FAM™ (6-carboxyfluorescein) 5' label (Applied Biosystems, Foster City, CA) were designed for *KRT10*, *KRT1*, and *KRT2e* using Perkin-Elmer Applied Biosystems Primer Express software (Table 1). The primers were designed so that the resulting amplicon crossed at least one intron-exon boundary. Because of the marked homology between keratins, the highly conserved regions of the keratin genes, particularly the trigger motifs and the H1 homologous subdomain, were avoided to help insure a high level of specificity.

A GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for quantitation. The amplification program consisted of an initial 95°C, 10-minute cycle followed by 40 cycles of 95°C 15-second denaturation and 60°C 1-minute annealing/extension. Samples were analyzed for the expression of KRT10 and an internal housekeeping gene, 18S rRNA (Applied Biosystems, Foster City, CA). The cycle threshold (C_T) values for each reaction were averaged. All genes were normalized to 18S rRNA, and the relative fold change was calculated with the formula: $X = 2^{-??Ct}$ where $??Ct = (C_{T,target} - C_{T,reference})_{sample} - (C_{T,target} - C_{T,reference})_{normal}$. Target refers to the gene of interest, and reference refers to the internal control gene. Sample refers to a cultured sample, and normal refers to normal epidermis (Lehmann *et al*, 2001)

Table 2. Sequences for TaqMan MGB probes and primers.

Gene name		**Primer sequence (5'-3')**	**Amplicon Size (bp)**
Keratin 10	Forward Reverse MGB Probe	CCTGCTTCAGATCGACAATGC ACCTCGTTCTCATACTTTAATCTGAAGTC AGGCTGGCAGCTGA	66
Keratin 1	Forward Reverse MGB Probe	CTCGGATGGATTCGGAATTG CCTCATACTTGTTCCGGTAATCTTC AGAACATGCAAGACCTG	65
Keratin 2e	Forward Reverse MGB Probe	CACGTGAAGAAGCAGTGTAAGAGTGT ACTGCGTGCTCTCCCTTCTG CAAGAAGCCATTGCAGAA	71

Results

Submerged normal canine keratinocytes failed to express K10

Moderate to intense staining for pancytokeratin (Fig 21A) and K14 (Fig 21B) were noted throughout the cytoplasm of greater than 90% of the cultured keratinocytes. The highest levels of fluorescence intensity for these two antibodies surrounded the nucleus, which reflects the normal density of keratin intermediate filament radiating from the nuclear membrane (Djabali, 1999). No fluorescence was detected in keratinocytes stained for K10

OT cultures of affected keratinocytes maintained the phenotypic alterations seen in vivo

No morphologic differences were noted between OT epidermis grown on acellular collagen rafts and rafts containing fibroblasts. The OT epidermis from normal dogs harvested on day 7 was 1-3 cell layers thick with a distinct basal and spinous cell layer, rare keratohyaline granules and no detectable stratum corneum (Fig 22A). The OT

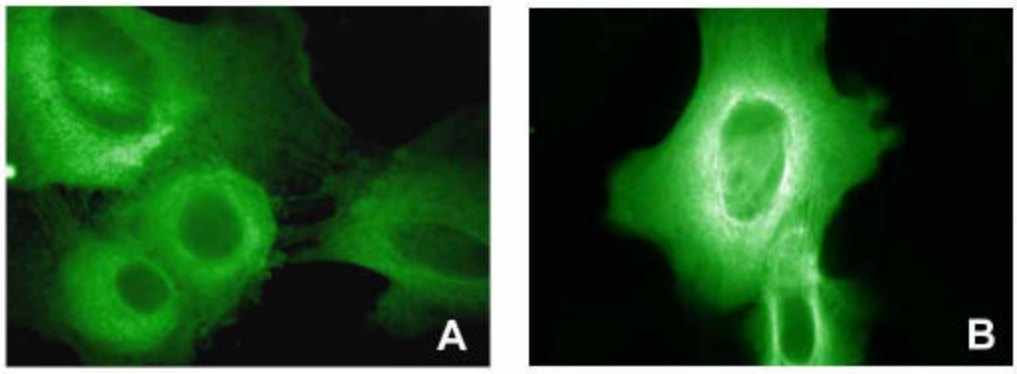


Figure 21. Fluorescent labelling of keratinocytes from normal dogs cultured under submerged conditions. Greater than 90% of the cells stained for pancytokeratin (A) and K14 (B). FITC pan-Ig. Original magnifications, X1000.

Epidermis from affected dogs was thinner with a disorganized basal layer and minimal differentiation (Fig 22B). On day 14, the normal OT epidermis was mildly hyperplastic with distinct basal, spinous, and granular cell layers and a thin, compact stratum corneum (Fig 22C). The affected OT epidermis on day 14 had a distinct basal layer, but keratohyaline granules were infrequently identified and cornification was not yet evident (Fig 22D). On day 21, the normal OT epidermis was markedly hyperplastic with a prominent, compact stratum corneum alternating with foci of parakeratosis (Fig 22E). The affected OT epidermis was mildly to moderately hyperplastic with focal areas of atypical cornification characterized by small exfoliating clusters of abnormally-shaped corneocytes (Figs 22F ,22G). Keratinocytes in the spinous and granular layers were frequently vacuolated with occasional intracytoplasmic eosinophilic inclusions and enlarged keratohyaline granules (Fig 22H).

On day 18, media in several of the culture inserts from one of the affected dogs contained large, exfoliated, ribbon-like aggregates of keratinocytes. Epidermis from one of the collagen rafts was harvested for histologic evaluation. Several large, intact epidermal clefts were noted in the superficial epidermis (Fig 23). The appearance of these keratinocyte aggregates in the culture media likely occurred secondary to epidermolysis in the suprabasal epidermis and mimicked the formation of superficial erosions seen clinically in affected dogs.

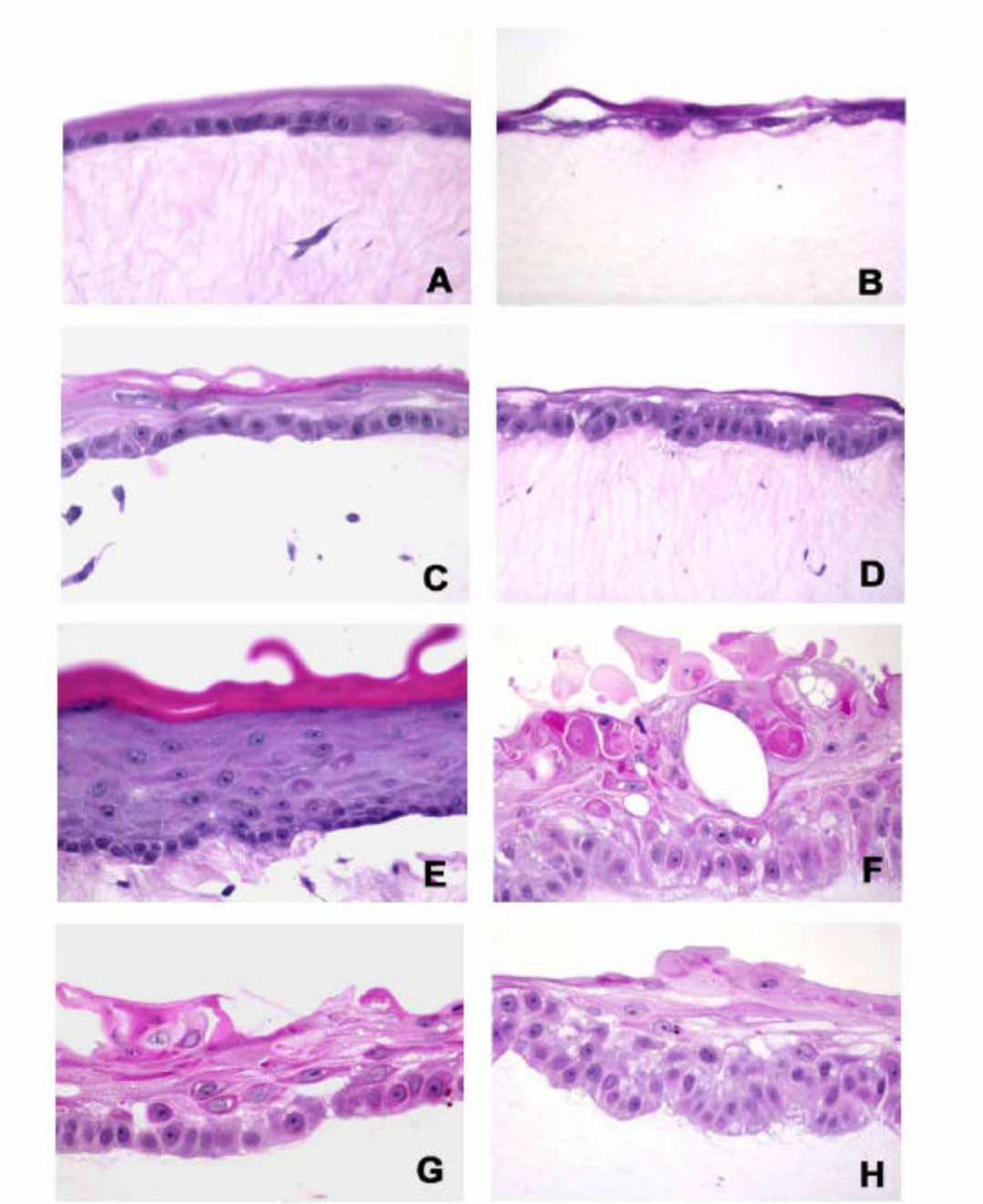


Figure 22. Normal and affected OT epidermis harvested at days 7, 14 and 21. (*A*) Normal OT epidermis at day 7 showed a well-defined basal layer and absence of cornification. (*B*) Affected OT epidermis at day 7 lacked an organized basal layer. (*C*) Normal OT epidermis at day 14 was mildly hyperplastic with evidence of cornification (*D*) Affected OT epidermis at day 14 contained a well-defined basal layer with no obvious cornification (*E*) Normal OT epidermis at day 21 was markedly hyperplastic with prominent cornification and foci of parakeratosis (*F*,*G*) Affected OT epidermis at day 21 was moderately hyperplastic with foci of irregular cornification (*H*) Affected OT epidermis at day 21 displayed prominent vacuolation, occasional intracytoplasmic eosinophilic inclusions, and several enlarged keratohyaline granule

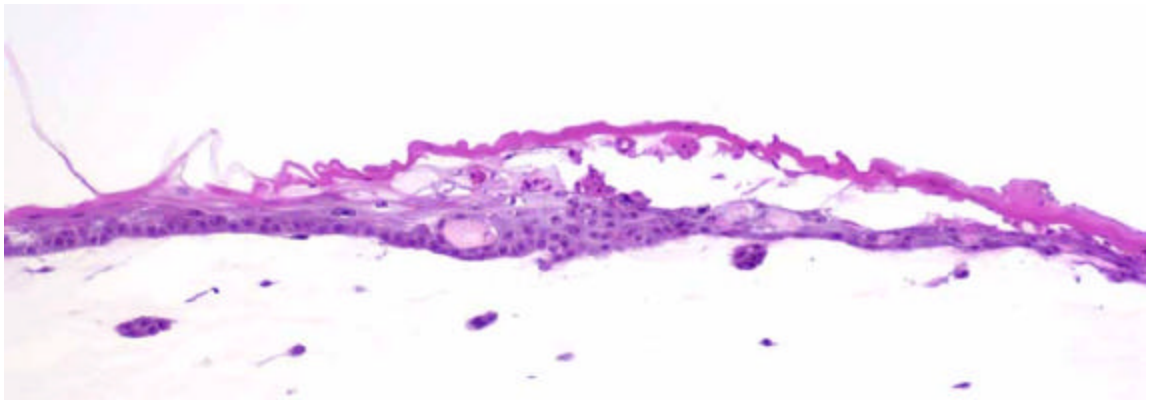


Figure 23. An affected collagen raft at day 18 showed prominent cleft formation in the superficial epidermis. H&E. Original magnification, X400.

Normal OT keratinocytes expressed K10 whereas affected OT keratinocytes did not express K10

Immunohistochemical staining for K10 was performed on cultured epidermis from both normal and affected dogs that was harvested on days 7, 14 and 21. Normal and affected OT epidermis failed to demonstrate K10 staining on day 7. Very light and patchy K10 staining was detected in the most superficial layers of normal OT epidermis

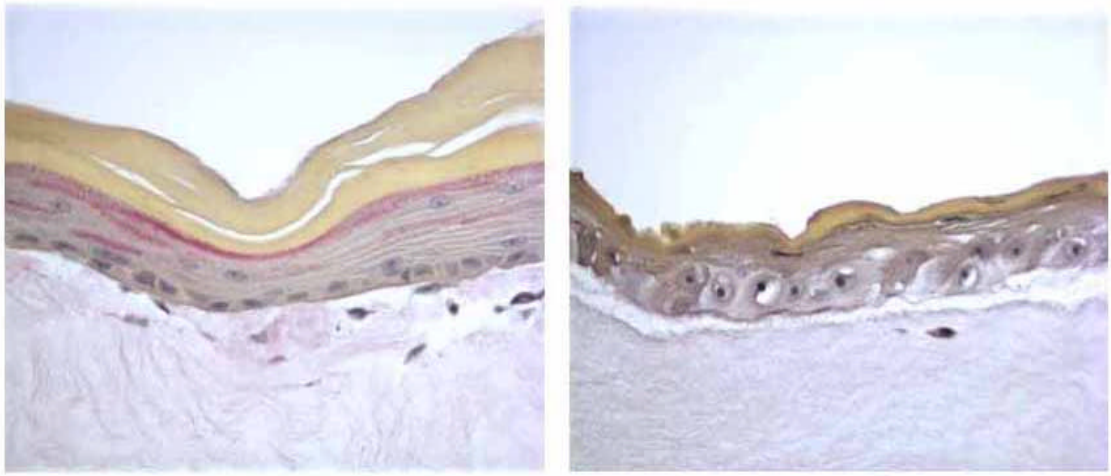


Figure 24. Immunohistochemical staining for K10. (*A*) At day 21, the normal OT epidermis showed mild to occasionally moderate, multifocal, intracytoplasmic staining for K10 in the superficial epidermis. (*B*) The affected OT epidermis lacked K10 staining. Alkaline phosphatase, Mayer's hematoxylin counterstain. Original magnifications, X400.

On day 14 while no staining was detected in affected epidermis. On day 21, normal OT epidermis displayed light to occasionally moderate K10 staining multifocally throughout the upper cell layers (Fig 24A). K10 staining was not identified in the affected OT epidermis (Fig 24B). A previous study involving keratins in the canine epidermis estimated the molecular weight of canine K10 at approximately 56 kDa (Walter, 2001). In this study, a band at 58 kDa was identified in the protein extracted from normal OT epidermis, but no band of similar size was detected in extracts obtained from affected OT epidermis (Fig 25).

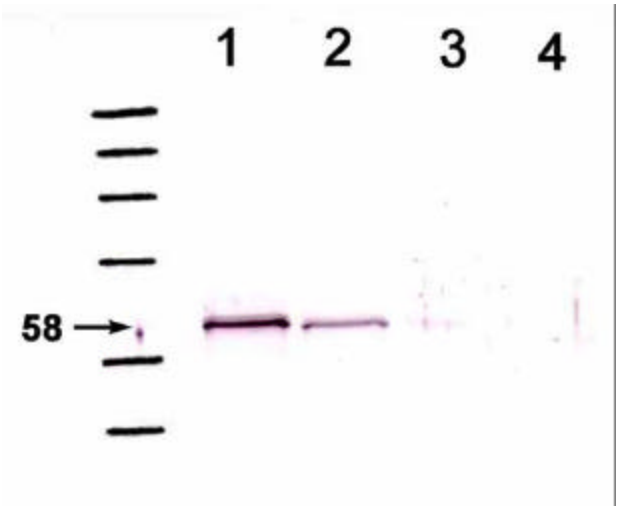


Figure 25. Immunoblot stained for K10. Lanes 1 and 2 contain protein extracted from normal OT epidermis that displayed a distinct band at approximately 58 kDa. No bands were present in lanes 3 and 4 which contain protein extracts from affected epidermis

Affected dogs displayed a marked decrease in KRT10, a marked increase in KRT2e and minimal increase in KRT1

Each real-time reaction was performed in triplicate. Two separate runs were performed on each RNA sample obtained from epidermis for a total of 6 values per sample. One run only was performed on each RNA sample obtained from OT keratinocytes for a total of 3 values per sample. The fold change for each sample is provided in table 2. On average, real-time RT-PCR quantitation showed a 31-fold decrease in *KRT10*, a 1.75-fold increase in *KRT1*, and a 136-fold increase in *KRT2e* between normal and affected epidermis. OT keratinocytes from normal and affected dogs also exhibited a decrease in *KRT10* expression, but to a much larger degree (241-

fold) than the epidermal samples. In contrast to *in vivo* epidermis, cultured keratinocytes displayed a marked decrease in *KRT1* (31-fold) and *KRT2e* (1467-fold). The amount of *KRT10* message was approximately 246-fold less in OT keratinocytes of normal dogs as compared to normal epidermis (data not shown).

Table 3. Fold change in mRNA expression between affected and normal dogs *in vivo* and *in vitro.*

Fold change for each sample as compared to the average of 4 normal samples in the same experimental system (skin or culture)						
	KRT10		***KRT1***		***KRT2e***	
	Epidermis	**Culture**	**Epidermis**	**Culture**	**Epidermis**	**Culture**
N1	-0.16	-1.08	0.17	3.4	1.62	2.1
N2	0.38	4.52	-0.27	3.0	2.72	4.7
N3	0.37	-1.20	1.56	-2.3	-1.31	-7.9
N4	-0.58	-3.52	-0.54	-9.3	0.67	-2.5
A1	-32.27	-147.03	1.39	-11.7	119.4	-1360.6
A2	-26.95	-335.46	1.56	-50.5	397.9	-1573.8
A3	-42.57		1.66		22.0	
A4	-22.04		2.39		5.43	
Average fold change of affected dogs as compared to normal dogs						
	-31	-241	1.75	-31	136	-1467

The numerical value for each dog is the average of 6 replicates for skin samples and 3 replicates for culture samples; N=normal; A=affected

Discussion

Conventional submerged keratinocyte cultures lack the stratification, vertical cell-cell contacts and cell-matrix interactions required for complete epidermal differentiation and formation of a stratum corneum (Merne and Syrjänen, 2003). Numerous *in vitro* experimental techniques have been developed that recreate the three-dimensional structure of the epidermis. To ensure complete differentiation, the OT culture system must provide a dermal equivalent, an air-liquid interface and exogenous growth factors.

The importance of mesenchymal interactions to normal epidermal genesis has necessitated the development of reconstructed dermal equivalents on which keratinocytes can proliferate and differentiate (Merne and Syrjänen, 2003). Traditionally, dermal equivalents have consisted of contracted collagen gels that contain living or irradiated fibroblasts (Bell *et al*, 1983; Parenteau *et al*, 1992), allogenic cadaver dermis (Parenteau *et al*, 1991) or de-epidermized dermis (Rosdy and Clauss, 1990; Mak *et al*, 1991; Fartasch and Ponec, 1994). The fibroblasts in these equivalents are a source of endogenous growth factors; however, additional exogenous growth factors added to culture media are often necessary for complete differentiation. Occasional studies have also described epidermal reconstruction directly on collagen-coated culture dishes or membranes without a dermal equivalent (Noél-Hudson *et al*, 1995; Williams *et al*, 1988; Ohsawa *et al*, 1999; Van Dorp *et al*, 1999) or on acellular collagen gels (Fartasch and Ponec, 1994). Recently, synthetic basement membrane preparations that polymerize to form a biologically active membrane have become commercially available. These preparations are extracted from the Engelbreth-Holm-Swarm mouse sarcoma that

contains abundant quantities of extracellular matrix proteins (laminin, collagen IV, entactin, heparan sulfate proteoglycan, and others) and numerous growth factors (Kleinman *et al*, 1982).

In addition to the appropriate dermal equivalents and/or substrates, proliferation of keratinocytes at an air-liquid interface that exposes the external surface of the epidermis to the air while allowing nutrients to be delivered from a lower chamber has proven critical for differentiation. Many commercially available culture systems simplify this process by providing specialized permeable inserts for culture dishes (Kondo *et al*, 1997; Nowinski *et al*, 2002; Suhonen *et al*, 2003). Although studies on keratinocytes grown under conventional, submerged culture conditions do demonstrate some biochemical markers of differentiation, more complete formation of a moderately permeable stratum corneum is dependent upon the presence of the air-liquid interface (Mak *et al*, 1991). Recent research has shown that air exposure is essential for barrier formation by maintaining an essential calcium gradient throughout the epidermis. A high level of calcium is required in the granular cell layer for complete differentiation and barrier formation. High humidity and moisture decrease the calcium in the granular cell layer and thus inhibit differentiation (Elias *et al*, 2002a; Elias *et al*, 2002b). Reduced humidity has been reported to improve cornification in organotypic culture systems (Supp *et al*, 1999).

Although a previous report has described canine keratinocytes grown under submerged conditions to be mildly stratified (Wilkinson *et al*, 1989), the normal Norfolk terrier keratinocytes grown under submerged conditions in this study remained in a

monolayer and failed to express K10, a marker for terminal differentiation. The culture conditions established for the OT culture of both normal and affected keratinocytes in this study were based upon two previously described reports of canine keratinocytes grown at the air-liquid interface (Jakic-Razumovic *et al*, 1994; Suter *et al*, 1991).

Canine keratinocytes display many similarities to human keratinocytes in culture; however, several notable differences exist. Recent studies have demonstrated that adult human skin incubated in low-Ca^{2+} medium degenerates rapidly while high-Ca^{2+} conditions support differentiation and maintenance of normal architecture (Tavakkol *et al*, 1999). Conversely, normal differentiation of canine keratinocytes can occur during conditions that cause altered calcium homeostasis, and calcium supplementation is not required for differentiation in culture (unpublished data). The keratinocytes in this study were cultivated under low calcium conditions in both submerged and organotypic cultures.

Certain growth factors, particularly epidermal growth factor (EGF), inhibit keratinocyte differentiation. EGF and other ligands for the epidermal growth factor receptor (EGFR) stimulate keratinocytes to proliferate, migrate and degrade extracellular matrix components (Pasonen-Seppänen *et al*, 2003). In addition, activation of the EGFR has anti-apoptotic effects and is essential for cell cycle activation (Jost *et al*, 2000). These proliferative effects inhibit differentiation and decrease expression of terminal differentiation markers, particularly K10 and filaggrin (Piepkorn *et al*, 1998; Pasonen-Seppänen *et al*, 2003; Piepkorn *et al*, 2003).

While human keratinocytes may be effectively cultured in the absence of EGF, long-term cultivation of canine keratinocytes depends upon the presence of high levels (typically 10ng/ml) of EGF and cholera toxin (Wilkinson *et al*, 1989). In one study, canine keratinocytes failed to grow beyond 2 passages in the absence of growth factors (Wilkinson *et al*, 1987). Initially, passage 2 keratinocytes from normal Norfolk terriers in this study were cultivated in slowly decreasing concentrations of EGF in an attempt to condition the keratinocytes to EGF-depleted conditions prior to growing them at the air-liquid interface. The keratinocytes did not tolerate EGF depletion and failed to proliferate efficiently. Complete growth arrest was apparent after 2-3 passages. This strict requirement for growth factor supplementation, particularly EGF, was problematic in establishing canine OT cultures that fully-differentiated and expressed markers of terminal differentiation at levels equivalent to keratinocytes *in vivo*.

OT cultures of naturally occurring mutations in superficial keratins in any species have been rarely described. The literature contains a single report describing an *in vitro* model of EHK in humans (Chipev *et al*, 1996) and a recent study failed to reproduce the EHK phenotype *in vitro* (El Ghalbzouri *et al*, 2003). The keratinocytes obtained from both normal and affected Norfolk terrier dogs in this study were successfully cultivated in both submerged and OT cultures, but the phenotype was only defined using the latter method.

In general, the epidermis generated through OT culture of normal Norfolk terrier keratinocytes morphologically resembled normal *in vivo* epidermis as evidenced by the histologic appearance of the basal, spinous and granular cell layers and the formation of

a stratum corneum. The major difference between the normal OT cultured epidermis and normal skin was the pattern of differentiation. At day 7, the OT cultured epidermis consisted of 1-4 nucleated cell layers, which is the normal thickness of canine epidermis, but lacked cornification. A well-formed stratum corneum was not present until day 21 when the epidermis was moderately hyperplastic. The prominent hyperplasia is likely associated with the upregulation of K16 (a marker for hyperproliferation) in growth factor supplemented cultures, particularly those supplemented with EGF (Gibbs *et al*, 2000; Wang and Chang, 2003).

Immunohistochemical staining for K10 of OT cultured keratinocytes from normal dogs revealed light, patchy K10 expression compared to normal canine epidermis, where K10 is expressed diffusely throughout the suprabasal layers. This pattern is similar to that described for human keratinocytes cultured under high concentrations of EGF (2-20ng/ml) (Jost *et al*, 2000). The decreased level of K10 expression in OT cultured keratinocytes as compared to normal skin biopsy samples examined by immunohistochemistry, immunoblot and real-time PCR analysis suggested that culture conditions required for viability of canine keratinocytes at an air-liquid interface prevented optimal keratinocyte differentiation *in vitro*. Optimization of culture conditions with alternative growth factors to EGF and decreased humidity may improve results and more accurately simulate the differentiation that occurs *in vivo.* 21 lacked the well-formed compact appearance seen in the OT epidermis from normal dogs. As expected from immunohistochemical staining of skin biopsy samples, the OT

keratinocytes from affected dogs failed to express K10 in all layers of the epidermis, and showed no detectable level of KRT10 expression.

Quantitation of *KRT1* and *KRT2e* revealed divergent trends between epidermis and cultured keratinocytes. Affected epidermis showed a slight increase in *KRT1* despite a marked decrease in its preferred dimerization partner, *KRT10*. This correlated with previous findings from a keratin-10-null mouse model that created a mild EHK phenotype in which K1 protein levels were slightly increased (Reichelt *et al*, 2002). This trend reflects the classical promiscuity of keratins in which a specific keratin can form a novel dimer in the absence of its natural partner. In this case, K1 is likely forming a novel keratin complex with either K14 or K16 (Reichelt *et al*, 1999).

The normal amount and pattern of K2e expression in the epidermis of dogs is not known. Murine K2e is reported to be much more abundant than human K2e, and further studies are required to determine if canine K2e parallels haired mouse or sparsely-haired human epidermis (Herzog *et al*, 1994). Assuming that canine K2e responds to stimuli for keratinocyte activation similarly to human K2e, the alterations in *KRT2e* expression both *in vivo* and *in vitro* parallel findings reported for hyperproliferative cutaneous lesions in humans (Bloor *et al*, 2003). In this report, increased K2e expression is correlated with mild to moderate keratinocyte activation, while cells fail to express K2e in conditions inducing marked activation. The gene expression data in our report show a similar trend with marked increase in *KRT2e* in affected epidermis that is likely mildly to moderately activated as opposed to the

marked keratinocyte activation of cultured epidermis associated with a marked decrease in *KRT2e*.

Despite evidence of delayed differentiation compared to normal OT epidermis, the OT epidermis from affected dogs largely recreated the histologic alterations described for this Norfolk terrier keratinization defect (Barnhart *et al*, 2004). One difference was the lack of a prominent granular cell layer, which may reflect incomplete differentiation; however, under-representation of the granular layer is commonly noted in organotypic cultures of normal keratinocytes. In addition, cytoplasmic keratin aggregates were seen less frequently in the affected OT epidermis.

In summary, this study demonstrated that diseased keratinocytes obtained from Norfolk terrier dogs with a heritable K10 defect may be successfully cultivated under OT conditions while preserving disease phenotype. Despite preservation of the disease phenotype, the decreased protein and gene expression levels for K10 and KRT10 in normal cultured keratinocytes as compared to normal dog skin indicated that terminal differentiation of the keratinocytes was altered. Changes to the culture system such as alternative growth factors and decreased humidity may improve *in vitro* differentiation.

Additionally, this study may provide an experimental model for research involving treatment of recessive keratin defects of the epidermis. All of the known mutations for K10 or K1 act in a dominant negative manner. Gene therapy for dominant diseases requires inactivation of the mutated gene while in most cases, reexpression of the mutated gene in recessive diseases is required to abrogate clinical signs (Uitto *et al*, 2000). Consequently, the Norfolk terrier model may not be useful for therapeutic studies

of EHK; however, several recessive mutations have been documented in another type I keratin, K14, that result in EBS (Hovnanian *et al*, 1993; Chan et al, 1994; Rugg *et al*, 1995; Jonkman *et al*, 1996; Corden *et al*, 1998; Batta *et al*, 2000; Yasukawa *et al*, 2002). Both EHK and EBS are generalized epidermolytic diseases and successful therapeutic modalities developed for one could be directly applied to the other.

CHAPTER IV

VALIDATION OF INTERNAL CONTROLS FOR RELATIVE mRNA QUANTIFICATION BY REAL-TIME RT-PCR IN CANINE EPIDERMIS

Introduction

"Housekeeping genes" are functionally defined as those genes that are constitutively expressed to maintain a basic cellular function (Warrington *et al*, 2000). These genes are frequently selected as internal controls for mRNA quantitation experiments because they are ubiquitously expressed and present at relatively constant levels. An ideal "housekeeping gene" for a specific experiment is one that is coexpressed with the target genes but not transcriptionally regulated during all stages of development regardless of tissue or experimental treatment. (Tricarico *et al*, 2002; Schmid *et al*, 2003). Published gene expression studies of human and mouse epidermis have typically utilized ß-actin, a cytoskeletal protein or glyceraldehydes-3-phosphate dehydrogenase (GAPDH), an enzyme of glycolysis.

More recently, eukaryotic 18S rRNA has been employed as an internal control in many studies involving epidermis and other tissues. Although this gene is considered an appropriate control for many experimental conditions, it has the major limitation of not being retained during RNA amplification. This procedure is being used with increasing frequency to overcome the limitation of low RNA yields in gene expression studies. Consequently, it is often necessary to utilize an alternative internal control.

Another gene commonly selected as an internal control for RNA quantitation studies is cyclophilin A (Feroze-Merzoug *et al*, 2002). Early studies on cyclophilin in the skin suggested a similar level of expression in both normal and diseased tissues (Griffiths *et al*, 1990; Chatellard-Gruaz *et al*, 1994). Recent analysis has shown that cyclophilin is expressed more consistently than most standard housekeeping genes during normal human keratinocyte differentiation and differentiation altered by replication of human papillomavirus, and therefore provides increased reliability in data obtained from real-time RT-PCR (Steele *et al*, 2002). However, cyclophilin may not always serve as the best internal standard for gene expression analysis in the skin as it has been shown to be significantly increased in biopsies from human psoriatic epidermis as compared to normal (Torma *et al*, 2000).

The purpose of this study is to evaluate the suitability of three housekeeping genes: GAPDH, cyclophilin and 18S rRNA for use in experiments involving both *in vivo* and *in vitro* analysis of normal and diseased canine keratinocytes.

Materials and methods

Sample groups and sample acquisition

Keratinocytes were obtained from 6mm skin punch biopsy samples from 11 dogs representing five different breeds: Labrador retriever (1), standard poodle (1), Norfolk terrier (NFT) (6), mixed breed dog (1) and Jack Russell terrier (JRT) (2). The retriever, poodle, mixed breed dog, 4 NFTs and 1 JRT had grossly normal skin. Two dogs were NFTs affected with a heritable keratinization defect (Barnhart *et al*, 2004) and one of the

NFTs with grossly normal skin was a carrier of this same disease. The final dog was a JRT affected with a heritable form of ichthyosis resembling lamellar ichthyosis in humans (Lewis *et al*, 1998). Primary keratinocyte cultures were established from 6 normal and 2 affected dogs as previously described (Suter *et al*, 1987), and organotypic cultures were established from 3 normal NFTs, one affected NFT, one normal JRT and one affected JRT (unpublished data; Jakic-Razumovic *et al*, 1994). A list of the samples used for this study is provided in table 4.

Table 4. Samples used for this investigation based on animal, clinical signs and whether sample was obtained from culture or skin biopsy. From top to bottom, the groups are ordered from least to most completely differentiated.

Dog #	Breed	Disease Statues	Submerged Culture	Organotypic Culture	Biopsy
1	Norfolk terrier	Normal	X		
2	Standard poodle	Normal	X		
3	Jack Russell terrier	Normal	X		
4	Labrador retriever	Normal	X		
5	Norfolk terrier	Normal		X	
6	Norfolk terrier	Normal		X	
7	Norfolk terrier	Normal		X	
8	Jack Russell terrier	Normal		X	
9	Norfolk terrier	Affected		X	
10	Norfolk terrier	Normal			X
11	Mixed breed	Normal			X
12	Norfolk terrier	Carrier			X
13	Norfolk terrie r	Affected			X
14	Jack Russell terrier	Affected			X

RNA extraction and quantitation

Skin biopsy samples were immediately frozen at –80°C in RNA Later (Ambion, Austin, TX). Organotypic cultures of keratinocytes were performed from a modified version of Jakic-Razumovic et al, 1994 and removed at 21 days from their underlying matrix by gentle scraping after 1 hour of trypsinization at 37°C and stored in RNALater at –80°C. Submerged cultures of keratinocytes were performed as previously described (Wilkinson *et al*, 1987), and keratinocytes were harvested at passages 3-5 by trypsinization (0.25% trypsin/1 mM EDTA) for 8-10 minutes at 37°C. Upon thawing, total RNA was immediately extracted from the cultured keratinocytes and the epidermis was carefully trimmed from the dermis and homogenized. Total RNA was isolated with an RNAqueous-micro kit (Ambion, Austin, TX) and treated with DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. RNA was quantitated with RiboGreen (Molecular Probes, Eugene, OR) and stored at –80°C.

Primer design and optimization

Primers were designed for 3 "housekeeping" genes; 18S rRNA, GAPDH and cyclophilin A (CYC). Primers sets were designed using Primer Express 1.0a software (PE Applied Biosystems, Foster City, CA). Forward and reverse primer concentrations were progressively diluted (300nM, 100nM, 30nM) to determine the optimal concentrations for SYBR Green PCR (Table 5). The reactions were performed on a GeneAmp 9600 thermocycler coupled with a GeneAmp 5700 sequence detections system (PE Applied Biosystems). The PCR reaction conditions were 48°C for 30

Table 5. Primer sets for selected housekeeping genes.

Gene	Primer Sequence	Accession Number	Final concentration of each primer
18S rRNA	F: CACATCCAAGGAAGGCAGCA R: TTTTCGTCACTACCTCCCCGG	337376 (Human seq)-Homologous to rat, pig, mouse, horse	40 nM each
GAPDH	F: GCCTCCTGCACCACCAACTG R: CCTCCACGATGCCGAAGTG	9247083	400 nM each
Cyclophilin A	F: ACGATGTTCATGCCCTCCTT R: ACCGCCAAGACTGAGTGGTT	AF243140	300 nM each

minutes, 95°C for 10 min, followed by 40 cycles consisting of a denaturing period at 95°C for 10s and an extension period of 60° for 1 min. A post-PCR dissociation curve was run according to manufacturer's directions.

SYBR green RT-PCR

A one-step RT-PCR amplification kit (SYBR Green PCR Master Mix and RT-PCR, PE Applied Biosystems) was used to generate all products. Reactions (25 ? l) consisted of 1X SybrGreen PCR Master Mix, 25 ng total RNA, 6.25U MultiScribe Reverse Transcriptase, 10U Rnase Inhibitor, and DEPC-treated deionized water as recommended by the manufacturer. Each assay was performed in triplicate under the PCR conditions described above. All reactions were then duplicated; thus, generating six individual data points for each primer set and sample. The average Ct value was normalized to 18S rRNA, and the relative fold change was calculated with the formula: $X = 2^{-??Ct}$ where $??Ct = (C_{T,target} - C_{T,reference})_{sample} - (C_{T,target} - C_{T,reference})_{normal}$. Target

refers to the gene of interest, and reference refers to the internal control gene. Sample refers to a cultured sample, and normal refers to normal epidermis (Lehmann *et al*, 2001)

Data analysis

All graphs were generated with the GraphPad Software program. A Spearman correlation coefficient was used to determine whether GAPDH or CYC positively correlated with 18S rRNA.

Results

Figure 26 displays the average of the 6 replicates for each of the fourteen selected keratinocyte populations. The samples were arranged in order from least differentiated to most differentiated. The degree of differentiation was based upon cell morphology with the least differentiated tissues lacking stratification and cornification, and the most differentiated having complete stratification and normal or excessive cornification.

Graphical representation of the average of the 6 replicates for each sample showed only slight variability for cyclophilin and 18S rRNA while GAPDH displayed a much higher degree of variability. Additionally, both CYC and GAPDH showed a slight trend toward increased Ct values in conjunction with increased differentiation and consequently less proliferation.

Patients were then grouped according to disease status (normal, carrier, affected) and experimental system (adherent cell culture, organotypic cell culture, skin biopsy sample) (Table 4).

Replicates from all individuals within each sample groups were averaged. The positive correlation between the Ct values for CYC and GAPDH and the level of keratinocyte differentiation was again evident while the Ct values for 18S rRNA remained relatively constant (Fig 27). Because 18S rRNA accounts for approximately 70% of all RNA present in a biological system, it is often used to estimate the total amount of RNA present. Based on this assumption, the ? Ct values between CYC-18S and GAPDH-18S were calculated. The ? Ct values again demonstrated a trend toward increased Ct values for increasingly differentiated keratinocyte populations (Fig 28).

The correlation between the 6 replicates for each of the housekeeping genes was evaluated with a Spearman non-parametric correlation and a significant positive

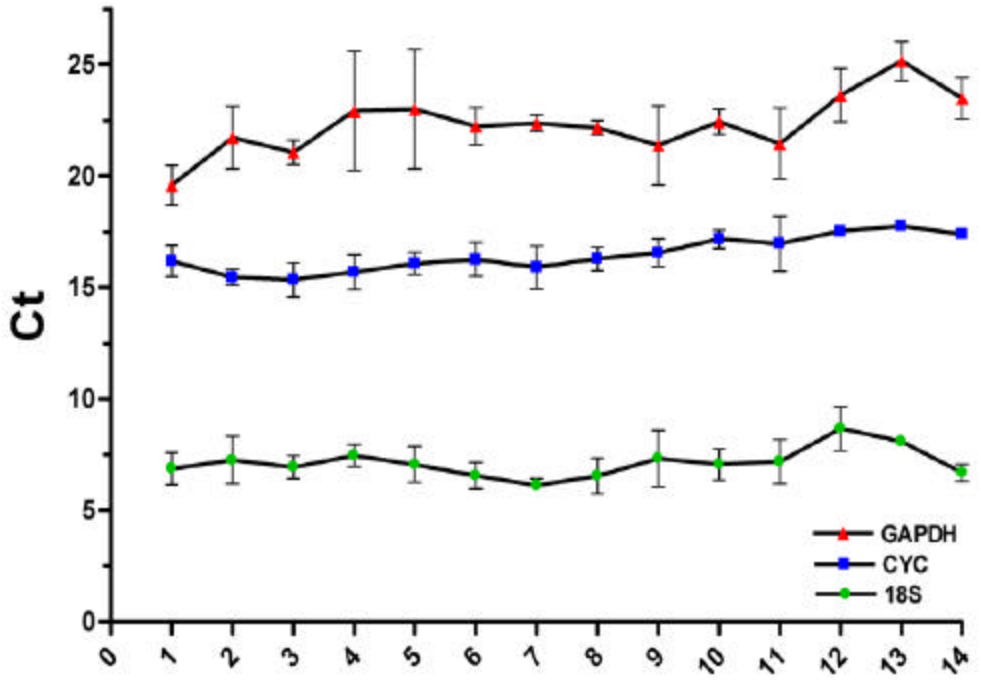


Figure 26. The average cycle threshold (Ct) value of 6 replicates for each of the 14 dogs. Error bars represent the standard deviation of the 6 replicates.

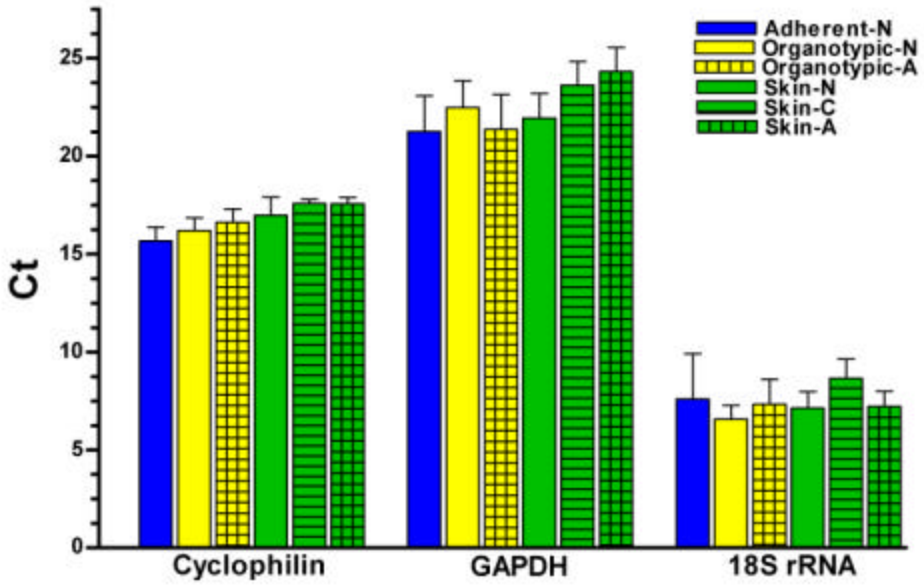


Figure 27. The average Ct values for each of the 6 groups of keratinocyte populations. From left to right, keratinocyte populations are ordered from least differentiated to most differentiated. Error bars represent the standard deviation.

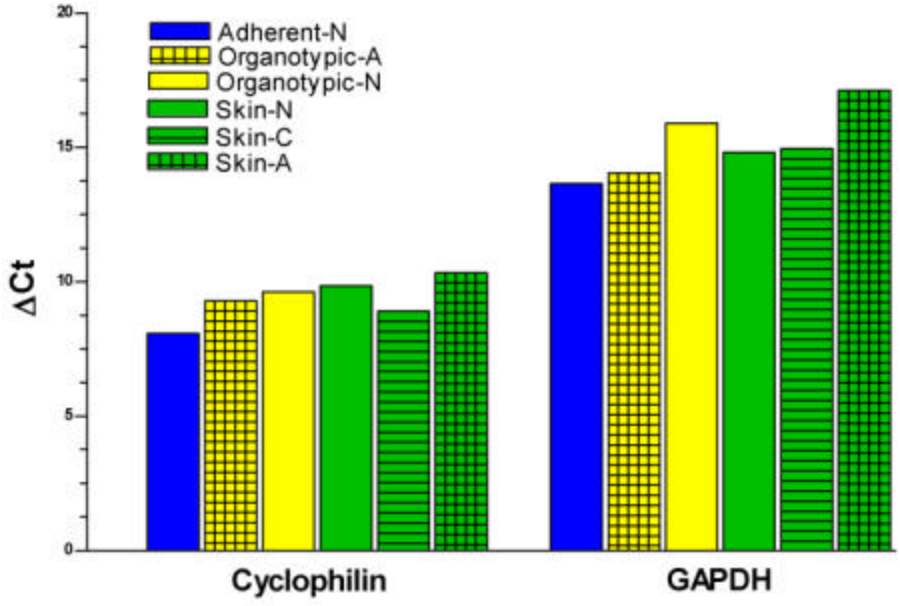


Figure 28. The ? Ct between 18S rRNA and CYC or GAPDH using the average Ct value for each group of keratinocytes. From left to right, keratinocyte populations are ordered from least differentiated to most differentiated.

correlation was determined for CYC and 18S rRNA with an r value of .2323 at a p-value of less than or equal to 0.05. A slight negative correlation was determined for GAPDH and 18S rRNA with an r value of -0.1074, but this was not significant at a p-value of less than or equal to 0.05.

Discussion

Selection of a control gene for real-time RT-PCR analysis is often based on the use of "traditional" housekeeping genes such GAPDH and B-actin without evidence that these genes are constitutively expressed in the tissue and/or experimental conditions being used (Schmid *et al*, 2003; Aerts *et al*, 2004). Previous studies have revealed many experimental conditions in which the transcription of internal control genes does not remain at a steady state level (Schmittgen *et al*, 2000). Significant variation in expression of control genes may also occur between individuals and samples taken from the same individual at different time points (Bustin, 2000). In addition, the suitability of a housekeeping gene varies greatly depending upon the tissue being studied (Blanquicett *et al*, 2002; Schmid *et al*, 2003). Because of these variations, confounding results may be obtained from identical experiments depending upon which gene is selected as the control (Glare *et al*, 2002; Tricarico *et al*, 2002). Several recent studies further contend that to avoid incorrect interpretation, gene expression should be related to at least 2 different housekeeping genes in parallel (Tricarico *et al*, 2002; Schmid *et al*, 2003). Whether one gene or multiple control genes are used, the selection of an appropriate gene ideally should be based upon controlled studies performed in each tissue. This is especially true for the epidermis, a tissue that

undergoes dramatic differentiation from basal cells to corneocytes under normal physiologic as well as pathologic conditions. (Bustin 2000; Bustin 2002).

Three genes that are often empirically selected as internal controls are eukaryotic18S rRNA, GAPDH and CYC. 18S rRNA is one of the most reliable and widely used controls. 18S rRNA has several unique intrinsic properties that enhance its desirability as a control. rRNA transcripts are generated by a distinct polymerase that is not used to synthesize mRNA; consequently, rRNAs are less likely to change under conditions that alter mRNA expression (Bustin, 2000). In addition, 18S rRNA has been shown to correlate highly with the amount of total RNA present in the sample and has a low turnover rate (Kim *et al*, 2003; Hashimoto *et al*, 2004). Despite these desirable properties, the use of rRNA as an internal control has three drawbacks; 1) rRNA transcription can be affected by biological factors and drugs, 2) it cannot be used for normalization when mRNA has been amplified as it is lost during purification and 3) it has a much higher abundance as compared to target mRNA (Bustin, 2000; Kim *et al*, 2003). Currently, 18S rRNA is one of the most common and reliable standards for quantitation of non-amplified RNA, but with the increasing need to amplify RNA for downstream applications, an alternative standard is often required.

Despite an abundance of literature demonstrating that GAPDH varies according to developmental stage, cellular proliferation and mitogenic stimulation including epidermal growth factor (Mansur *et al*, 1993; Calvo *et al*, 1997; Glare *et al*, 2002), GAPDH is still widely used as an internal control for experiments involving altered epidermal differentiation; however, in our study, GAPDH varied widely within and between experimental groups consistent with the preponderance of evidence in the literature that its

use as an internal standard is inappropriate in most experimental systems (Oliveira *et al*, 1999; Thellin *et al*, 1999; Bustin, 2000; Schmittgen and Zakrajsek, 2000; Wu and Rees, 2000; Glare *et al*, 2002; Rhoads *et al*, 2003; Schmid *et al*, 2003; Aerts *et al*, 2004).

Cyclophilins constitute an omnipresent protein family known as peptidyl prolyl isomerases that are expressed in most tissues and are found in all eukaryotic species (Schmid et al, 1993). They possess both sequence-specific binding and proline *cis-trans* isomerase activities which accelerate protein folding *in vitro* and enhance the immunosuppressive properties of cyclosporine A (Kern *et al*, 1995; Gothel *et al*, 1999; Steele, 2002). Cyclophilins have import roles in cell signaling, mitochondrial function, chaperone activity, RNA splicing, the stress response, gene expression, and the regulation of kinase activity (Takahashi, *et al*, 1989; Bukrinsky, 2002). Cyclophilin has been widely used as an internal control for gene quantitation, and a recent study suggested it is a reliable internal standard quantitation genes associated with epidermal differentiation (Steele *et al*, 2002)

To investigate the expression of "housekeeping" genes during epithelial differentiation, RNA was isolated from keratinocyte populations with either normal (skin biopsies), incomplete (submerged and organotypic cultures) and/or altered differentiation (keratinocytes from dogs with two different cornification disorders). Results of this study showed that 18S rRNA is a reliable internal control for cutaneous studies involving keratinocytes obtained from both *in vitro* and *in vivo* studies and diseases that involve altered differentiation. Although the Cts appeared to decrease slightly as keratinocyte populations became increasingly differentiated, the statistically significant positive

correlation between cyclophilin and 18S rRNA suggests that the use of cyclophilin as an appropriate internal standard is also valid for the samples analyzed in this study. However, cyclophilin, one of the initial targets of the immunosuppressive drugs cyclosporine A, FK506 and rapamycin, may have significant immunomodulatory effects, so its use as an internal reference in gene expression studies involving inflammatory diseases needs to be further investigated (Ivery, 2000).

CHAPTER V

DIFFERENTIAL GENE EXPRESSION IN NORMAL, HETEROZYGOTE AND AFFECTED NORFOLK TERRIER DOGS WITH A KERATIN 10 MUTATION

Introduction

Recently, a heritable defect in keratin 10 was described in Norfolk terrier dogs that had the unique feature of an autosomal recessive mode of inheritance (Barnhart *et al*, 2004; Credille, Br J Dermatol article submitted). The vast majority of heritable keratinization defects reported in people are autosomal dominant. Autosomal recessive keratin mutations in humans have been described but all involve the basilar keratins 5 and 14, (Hovnanian *et al*, 1993; Corden *et al*, 1998; Schuilengua-Hut *et al*, 2002; El Ghalbzouri *et al*, 2003; Lanschuetzer *et al*, 2003). Although a genetically modified mouse with a knockout mutation in keratin 10 has been developed (Reichelt and Magin, 2002), to date, spontaneous recessive keratin mutations have not been identified for keratins expressed in the superficial epidermis; keratins 1 (K1), 2e (K2e), 9 (K9) and 10 (K10), in any species other than the dog. Thus, the recessive mutation in the Norfolk terrier provides a unique opportunity to investigate gene alterations associated with a keratinization defect in a natural disease model. In addition, gene expression changes that occur in heterozygous dogs may provide insight into the molecular mechanisms that allow them to remain phenotypically normal.

One of the goals of this study was to determine the global gene expression changes of the epidermis that occur in normal, heterozygous and affected dogs through cDNA microarray analysis. As is the case for most mammalian species, a very small portion of the canine genome is annotated and few species-specific products are available for molecular genetic research. Until recently, a canine-specific cDNA microarray was not commercially available, and the cost of custom-made microarrays is prohibitively expensive for most canine genetics laboratories (Balkovetz *et al*, 2004). To address these limitations, several researchers have used cross-hybridization techniques that allowed pre-designed human microarrays to be probed with non-human cDNA (Cros *et al*, 1999; Huang *et al*, 2000; Medhora *et al*, 2002; Moody *et al*, 2002; Li *et al*, 2004). However, cross-species applications for hybridization technologies are still being validated and few controlled studies have investigated the feasibility of using human cDNA microarrays to define gene expression patterns in other species (Wang *et al*, 2004). In this study, we developed cross-species techniques that allowed canine cDNA to be hybridized to the human DermArray nylon membrane designed specifically for cutaneous research (Integriderm, Birmingham, AL).

Materials and methods

Sample collection and RNA preparation

Total RNA was obtained from 6mm skin punch biopsy samples from a total of 12 Norfolk terrier dogs. The dogs were determined to be normal, heterozygous or affected based upon a diagnostic PCR assay established at the Comparative Dermatology

Laboratory at Texas A&M University. Four dogs each were classified as normal, heterozygous or affected. The skin punch biopsy samples were obtained from the dorsolateral thoracic region, placed in RNA later (Ambion, TX) and stored at -80C.

RNA isolation

The epidermis was excised from the underlying dermis for each biopsy sample and briefly homogenized. Total RNA was extracted with an RNAqueous-micro kit (Ambion, Austin, TX) and treated with DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. The RNA was quantitated with RiboGreen (Molecular Probes, Eugene, OR) and stored at –80C.

T7 amplification

Linear T7 amplification was performed using the MessageAmp kit (Ambion, Austin, TX). Briefly, first strand cDNA synthesis was performed on 100ng of DNA-Free treated total RNA from each of the 12 dogs. First strand synthesis was carried out by reverse transcription with an oligo (dT) primer containing a T7 RNA polymerase promoter sequence on the 5' end. Synthesis of the second strand to produce full length dsDNA was accomplished by combining dNTPs, DNA polymerase and Rnase H, then incubating for 2 hours at 16C. An equilibrated filter cartridge was used to purify the dsDNA. Anti-sense (aRNA) was synthesized from the dsDNA by T7 *in vitro* transcription using a solution containing dNTPs, T7 reaction buffer and T7 enzyme mix. This solution was incubated at 37C for 6-8 hours and then purified using an equilibrated filter cartridge.

Synthesis of labeled cDNA probes

The cDNA probes were reverse transcribed and labeled with 250ng of aRNA, AMV reverse transcriptase, dNTPs, and (a-P^{33})-labeled dCTP at 37C for 2 hours according to the manufacturers protocol (Invitrogen, Carlsbad, CA). The probes were purified using spin columns (Invitrogen, Carlsbad, CA), eluted in tris-edta (TE) at a pH of 8.0 and denatured at 99C for 4 minutes.

Hybridization of cDNA microarrays

DermArray (Integriderm, Birmingham, AL) nylon membranes were pre-hybridized with MicroHyb hybridization solution (Invitrogen, Carlsbad, CA) containing 5ug of poly dA (Invitrogen, Carlsbad, CA) and 5ug of Human Cot-1 DNA (Invitrogen) to prevent amplification of poly A tails and repetitive sequences. Pre-hybridization was performed for 2 hours followed by addition of the purified and denatured probe. Hybridization was carried out at 37C overnight for 16 to 18 hours using a hybridization kit containing an AMV reverse transcriptase (Invitrogen, Carlsbad, CA) and P^{33} radiolabelled dCTP. Following hybridization, the membranes were washed twice with 1X SSC, 1% SDS at 50C for 15 minutes followed by a final wash with 0.5X SSC, 0.5% SDS at 50C for 15 minutes. The DermArray membranes were exposed to a phosphorimager screen (Fuji, Stamford, CT) and processed with a BAS 1800 phosphorimager (Fuji, Stamford, CT). Data were exported as TIFF files.

All DermArray membranes were used 3-4 times, and following each usage, the membranes were stripped of probe by boiling in 0.5% SDS for 1 hour. After the first hour of stripping, the membranes were re-exposed to a phosphorimager screen to

determine if any residual probe was still bound to the membrane. An additional 30-60 minutes of stripping was often required. On average, membranes required 90 minutes of exposure to 0.5% SDS to completely remove the labeled probe.

Data analysis

The raw intensity signals for each gene and the average experimental background were determined using Pathways 4.0 software (Invitrogen, Carlsbad, CA). These data were then imported into Microsoft Excel spreadsheets for further calculations with the GeneSpring software, version 5.0 (Silicon Genetics, Redwood, CA). The average background for each array was subtracted from the raw intensity value for each individual spot on the array. After subtraction, genes that had values less than 100 were set to 100. Each subtracted value was then normalized to the median intensity of the membrane. For normalization between experimental conditions, the normalized value for each gene was then compared to the average intensity of the same gene from a control sample. The control sample for this study was the average gene expression of the four normal dogs.

Following normalization, genes were initially filtered based on confidence using the logarithm of the expression (signal/control) ratio with the cross-gene error model active. The cross-gene error model estimates precision by using measurement variation and sample-to-sample variation (Gana Dresen *et al*, 2003). This model makes two assumptions; 1) variability between replicates is similar for all genes with a similar expression level and 2) a general purpose array was used where most genes have little biological variability. All 4480 genes found on the DermArray membrane were

evaluated with this model. Only genes with a p-value of less than or equal to 0.05 were considered for further analysis.

Genes that were identified by the cross-gene error model as differentially expressed were further selected based on fold change. Genes displaying a greater than or equal to 2-fold increase in expression level or a lesser than or equal to 1.5-fold decrease in expression level were considered differentially expressed. Selecting genes based on a combination of fold change threshold and p-values largely reduced the number of false positives (Natale *et al*, 2003) but decreased the sensitivity of the microarray. Expression differences were evaluated between normal and affected dogs and normal and carrier dogs. For the purpose of this study, significant differences between carrier and affected dogs were not evaluated.

DermArray membranes contain 383 genes that are spotted in triplicate at different locations across the membrane. As previously suggested (Wang *et al*, 2004), the average standard deviation (SD) of all 383 genes for each of the 12 dogs in the study was calculated as an estimate of the degree of variability incurred by the lower stringency conditions required in cross-hybridization.

Validation of differentially expressed genes by quantitative real-time RT-PCR

Increased or decreased expression was confirmed for 8 selected genes. Seven genes were evaluated by real-time RT-PCR with SYBR green. One gene was validated by TaqMan® real-time PCR. The genes evaluated by real-time PCR with SYBR green all had partial sequence available. Cyclophilin A was used as the internal control. Primers sets were designed using Primer Express 1.0a software (PE Applied Biosystems, Foster City, CA).

Forward and reverse primer concentrations were progressively diluted (600nM, 300nM, 100nM, 30nM) to determine the optimal concentrations for SYBR Green PCR. A one-step RT-PCR amplification kit (SYBR Green PCR Master Mix and RT-PCR, PE Applied Biosystems, Foster City, CA) was used to generate all products. Reactions (25 ?l) consisted of 1X SybrGreen PCR Master Mix, 25ng total RNA, 6.25U MultiScribe Reverse Transcriptase, 10U RNase Inhibitor, DEPC-treated deionized water. The reactions were performed on a GeneAmp 9600 thermocycler coupled with a GeneAmp 5700 sequence detection system (PE Applied Biosystems, Foster City, CA). The PCR reaction conditions were 48°C for 30 minutes, 95°C for 10 min, followed by 40 cycles consisting of a denaturing period at 95°C for 10s and an extension period of either 60° or 62° for 1 min. A post-PCR dissociation curve was run according to manufacturer's directions. Each assay was performed in triplicate under the PCR conditions described above.

KRT1 was validated by TaqMan® real-time PCR because of the high level of homology within the keratin family. This method used 10ng of RNA/reaction and the TaqMan® One-step RT-PCR Master Mix reagents kit (PE Applied Biosystems, Foster City, CA). In addition to gene specific primers, the TaqMan reaction used a gene specific probe (KRT1 probe = 5'-CCTGCTTCAGATCGACAATGC-3') containing an MGBNFQ (Minor groove binder/Non-fluorescent quencher) 3' label and a 6FAM™ (6-carboxyfluorescein) 5' label (PE Applied Biosystems, Foster City, CA). Primer specifications for both SYBR green and TaqMan reactions are provided in Table 6.

Table 6. Primer sequences used for gene validation.

Gene name		Primer (5'-3')	Tm	Conc	Primer Addition	Size
MPHOSPH6	Forward	CCGAGCGCAAGACGAAA	62	300nM	1-step	66
	Reverse	AATCCAGTCCTCTTTGCATGAAC		300nM		
MGEA6	Forward	TGATTCATCTCTCCCTGCTGAA	60	300nM	2-step	86
	Reverse	GAAACAATGGACCTCTGATTGGA		300nM		
EGF	Forward	CTGAAACACACTCAGCCCATGTAC	60	300nM	2-step	73
	Reverse	GGCCGAAATCATGGTGTCA		300nM		
SLC25A5	Forward	AGGGCGCAAAGGAACTGACA	60	300nM	2-step	60
	Reverse	GCAATCTTCCGCCAGCAGTCA		600nM		
COL1A2	Forward	CATGCTCAGCTTTGTGGATACG	60	300nM	2-step	~70
	Reverse	GCAGTTGCCTCCTGTAAAGATTG		300nM		
SMPD2	Forward	GGCTGCTGGTGCTCCATCTA	60	300nM	2-step	62
	Reverse	GGCATGGAGATGGGTCACAT		300nM		
FVIII	Forward	ACAGCATCCGCAGCACTCTT	60	300nM	2-step	68
	Reverse	CAGCGGCATGCTGCAA		300nM		
KRT1	Forward	CTCGGATGGATTCGGAATTG	60	300nM	1-step	65
	Reverse	CCTCATACTTGTTCCGGTAATCTTC		300nM		
	MGB probe	AGAACATGCAAGACCTG		250nM		
CYC	Forward	ACGATGTTCATGCCCTCCTT	60	300nM	1-step	71
	Reverse	ACCGCCAAGACTGAGTGGTT		300nM		

Results and discussion

Visual appraisal of both canine and human cDNA hybridization on the nylon membranes demonstrated similar intensity and specificity (Fig. 29). The average standard deviation (SD) varied from 7.5 to 21.9% (Fig. 30) with a mean of 14.3% and is consistent with data acquired at Integriderm, Inc demonstrating that 20% SD in the expression of single genes frequently occurs between experiments that do not require cross-hybridization (Wang *et al*, 2004). Consequently, cross- hybridization between dogs and humans did not significantly alter the reproducibility of cDNA binding across multiple spots.

Affected vs normal Norfolk terriers

The gene list was initially filtered based on confidence (p=0.05) with the cross-

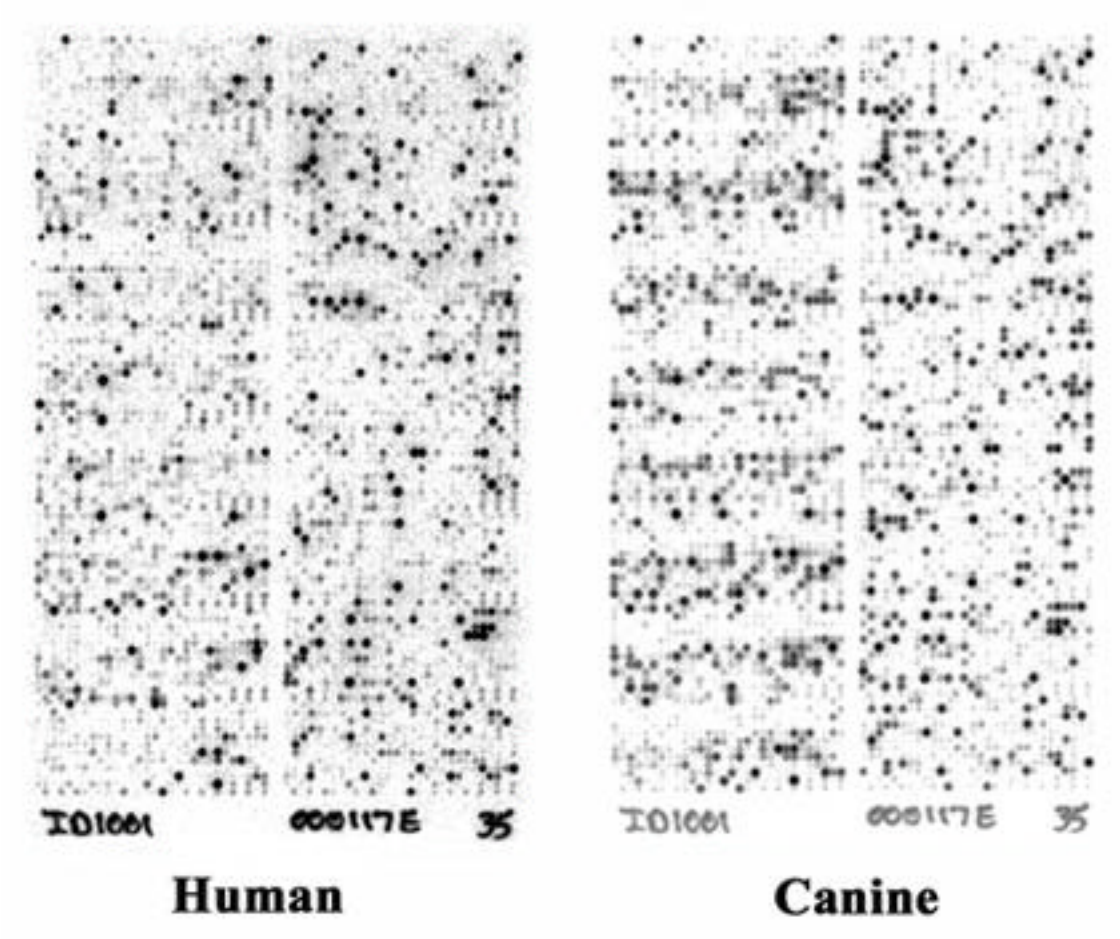


Figure 29. Comparison of human and canine hybridizations on DermArray membranes. Reduced stringency of hybridization and washing conditions for canine cDNA does not create a significant increase in background, and a large number of genes have a high level of homology and are able to bind to the human cDNA.

gene error model to obtain a list of 408 genes with significantly different levels of expression. From this subset, 320 genes were then selected based on either a 2-fold increase or 1.5-fold decrease in expression. Of these genes, 217 (68%) were upregulated and 103 (32%) were downregulated. The sequences of genes identified as ESTs, unnamed gene products or unnamed clones were analyzed for recent annotation through

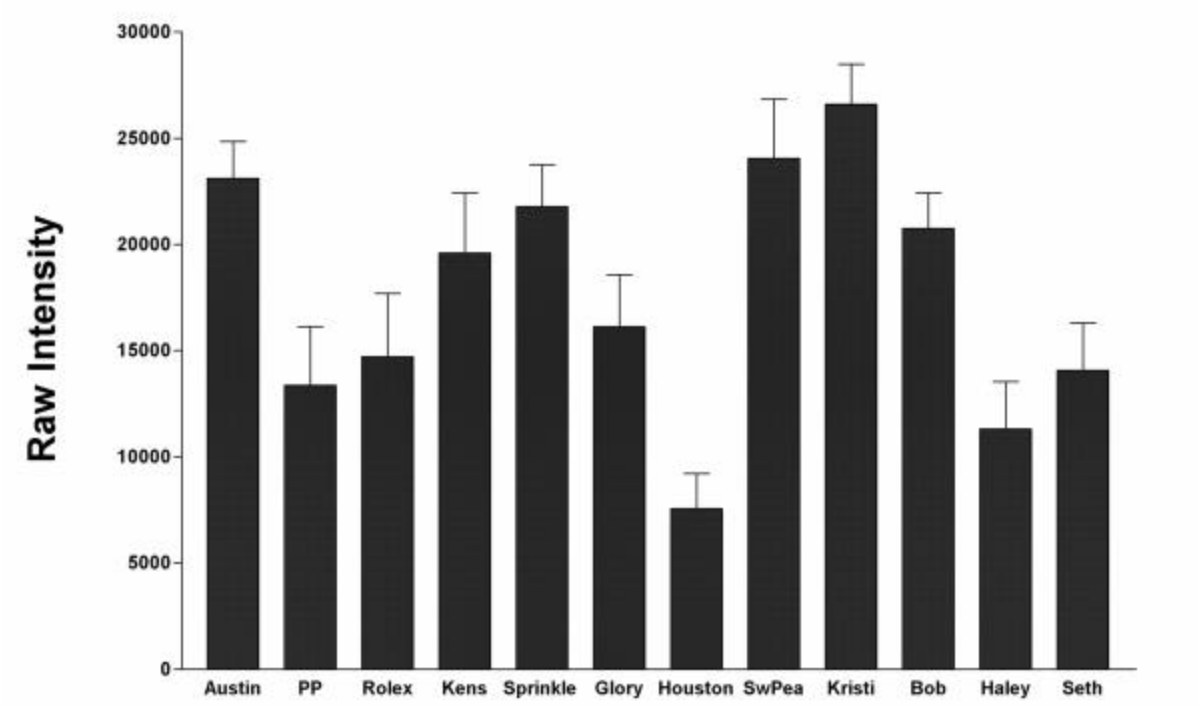


Figure 30. Average expression and standard deviation of the 383 genes spotted in triplicate for each sample dog.

NCBI Blast (ncbi.nlm.nih.gov/BLAST). Unidentified genes were then eliminated from further analysis. The general function of the remaining genes was determined, and genes with unknown function were also excluded from further analysis. Following annotation, 197 (123 upregulated and 74 downregulated) defined genes were identified for further analysis (Table 5). *Heterozygous (carrier) vs normal Norfolk terriers*

The same selection procedure that was used for affected dogs was used to compare the heterozygous carrier and normal dogs. The initial gene list based on confidence filtering (p=0.05) with the cross-gene error model contained 424 genes. This number was reduced to 298 genes based on either a 2-fold increase or 1.5-fold decrease in

expression. Of these genes, 222 (74%) were upregulated and 76 (26%) were downregulated. Following annotation, 173 (122 upregulated and 51 downregulated) defined genes were identified for further analysis (Table 7).

Differentially expressed genes present in both affected and heterozygous Norfolk terriers

Of the 408 differentially expressed genes in the skin of affected dogs based on confidence filtering, 88 (21%) of the genes were also differentially expressed in the heterozygous dogs. Of the 320 genes then selected based on fold change, 72 (23%) of the genes were also differentially expressed in the heterozygous dogs. Following annotation, 46 defined genes were identified as having differential expression in both disease states. All of the differentially expressed genes were positively correlated. No negatively correlated genes were identified (Table 7).

Microarray validation

To validate the microarray results, semiquantitative real-time RT-PCR was used with eight genes from different functional categories displaying variable fold changes. The magnitude of the fold change was typically greater in the upregulated genes and smaller in the downregulated genes as compared to real-time RT-PCR (Fig 31A,B).

Table 7. Functionally annotated list of the differentially expressed genes with known identity in affected, carrier and both groups.
*Genes listed in black = upregulated, red = downregulated

					Normalized values			
Gene description	**Function**	**Gene name**	**p-val**	**Fold ?**	**Aff**	**Carr**	**Nor**	**GenBank**
Apoptosis, stress and antioxidant								
Affected								
Thioredoxin-like 1	Exact function unknown	TXNL1	0.024	2.272	2.748	2.005	1.209	AA078976
ATX1 antioxidant protein 1 homolog (yeast)	Antioxidant against H202 and superoxide; copper chaperone	ATOX1	0.004	2.141	1.460	1.546	0.722	AA418694
Ectonucleoside triphosphate diphosphohydrolase 1 (CD39 antigen)	Depolarization causes endothelial superoxide production, which inhibits endothelial NTPDase-1 &enhances platelet aggregation	CD39	0.011	5.007	2.687	1.525	0.537	H13211
S100 calcium-binding protein A8 (calgranulin A)	Found in neutrophils, keratinocytes; function unknown	S100A8	0.004	5.115	5.008	0.977	0.979	AA086471
Programmed cell death 10	No specific function suggested	PDCD10	0.012	3.107	3.028	1.643	0.974	R68555
Heat shock 70kDa protein 12A	Increased in psoriasis; restricted to basal layer	HSPA12A	0.003	0.394	0.265	1.213	0.672	H17950
S100 calcium-binding protein A13	Undetermined function and tissue distribution	S100A13	0.046	0.606	0.600	0.394	0.991	AA070489
CD5 antigen-like	Scavenger activity, exact function not known	CD5L	0.049	0.135	0.076	0.464	0.564	AA677254
Carrier								
Tumor necrosis factor (ligand) superfamily, member 10	Expressed in most normal tissues, induces apoptosis in transformed & tumor cells, but doesn't kill normal cells	TNFSF10	0.035	4.632	1.116	2.275	0.491	H54629
Both								
TIA1 cytotoxic granule-associated RNA-binding protein-like 1	Apoptosis promoting, regulates alternative pre-mRNA splicing	TIAL1	0.013	2.071	1.930	1.846	0.892	N59426
Cell adhesion								
Affected								
Cell adhesion molecule w/homology to L1CAM	Keratinocyte growth factor related gene	CHL1	0.012	5.283	5.251	3.496	0.994	R40400
Cadherin 6, K-cadherin (fetal kidney)	No specific function known for the epidermis	CDH6	0.003	4.864	2.272	1.198	0.467	AA421819
Gap junction protein, beta 2, 26kDa (connexin 26)	Not expressed in normal human epidermis, upregulated in hyperproliferative epidermal states	GJB2	0.014	3.276	3.346	1.717	1.021	AA490466
Poliovirus receptor-like 1	Found in normal epidermis, colocalizes with E-cadherin, part of a novel cadherin-based cell-cell adherens junctions	PVRL1	0.047	2.151	2.154	1.410	1.002	AA911971
Tight junction protein 2 (zona occludens 2)	Colocalizes with zona occludens 1 at cell-cell borders primarily from the spinous to the granular layers	TJP2	0.003	0.079	0.061	0.198	0.769	W31391
Carrier								
Integrin, alpha 6	Interacts with ß4 integrin and is polarized on ventral side of basal cells where it mediates adhesion of laminin-5 and others	ITGA6	0.003	6.898	0.564	3.055	0.443	R43483
Fibrinogen, B beta polypeptide	Regulates cell adhesion and spreading, displays vasoconstrictor and chemotactic activities, and a mitogen for several cell types	FGB	0.006	5.457	7.141	4.843	0.888	H91714
Cadherin 11 (OB-cadherin, osteoblast)	Expressed in the hair follicle	CDH11	0.002	3.265	1.124	2.500	0.766	H96738
InaD-like protein	Localizes to tight junctions and apical epithelial membranes	INADL	0.042	0.529	0.619	0.334	0.631	AA005153

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold ?	Normalized values Aff	Carr	Nor	GenBank
Both								
Cadherin 13, H-cadherin or T-cadherin	Expressed only in basal keratinocytes, negative growth regulator of EGF, down-regulation leads to cancer	CDH13	1E-06		13.574	15.389	1.282	R41787
Cell cycle								
Affected								
IGF-II mRNA-binding protein 3	Interacts with MPHOSPH10, overexpressed in cancer	IMP-3	0.006	3.201	2.002	2.264	0.626	AA011347
M-phase phosphoprotein 6	RNA degradation	MPHOSP6	0.008	10.827	4.126	1.418	0.381	AA478525
Cell division cycle 16	Promotes anaphase	CDC16	0.006	4.276	4.292	1.880	1.004	AA410604
ZW10 (Drosophila) homolog, centromere/kinetochore protein	Important for proper chromosomal segregation	ZW10	0.022	4.580	2.510	1.640	0.548	AA599145
Kruppel-like factor 4 (gut)	Cell cycle checkpoint protein, prevents entry into mitosis following DNA damage, involved with epithelial differentiation	KLF4	0.047	2.191	2.104	1.674	0.960	H45668
Tuberous sclerosis 2	Prolonged S phase, increased apoptosis	TSC2	0.046	0.289	0.134	0.980	0.463	H37774
B-cell translocation gene 2	Negative growth regulator	BTG2	0.01	0.099	0.093	0.665	0.939	H69583
G0S2 putative lymphocyte G1/G2 switch gene	Function undetermined	G0S2	0.019	0.384	0.365	0.858	0.952	AA931758
Cell division cycle 45	Important in the early steps of DNA replication	CDC45	0.04	0.223	0.166	0.379	0.746	AA700904
Carrier								
Menage a trois 1 (CAK assembly factor)	Modulates CAK which regulates G1 exit; leads to proliferation	MNAT1	2E-04	8.110	0.560	5.090	0.628	AA481759
Cullin 1	Part of E3 ubiquitin ligase SCF	CUL1	0.012	4.469	0.720	2.148	0.481	AA486790
Cyclin-dependent kinase inhibitor 2A	Inhibits cell cycle G1 progression through the regulatory roles of CDK4 and p53	CDKN2A	0.003	2.329	0.668	2.223	0.954	AA877595
Budding uninhibited by benzimidazoles 1, beta	Mitotic checkpoint, prevents early separation of chromatids	BUB1B	0.007	4.670	2.391	6.987	1.496	AA488324
Glycoprotein (transmembrane) nmb	Found in some melanomas, may be assoc with growth delay	GPNMB	0.016	6.000	1.322	3.045	0.508	AA425450
Cyclin A2	Promotes G1/S and G2/M transition, found in all cells	CCNA2	0.022	2.833	2.096	1.678	0.592	AA608568
CDC20 cell division cycle 20 homolog	Required for nuclear movement prior to anaphase and chromosome separation	CDC20	0.04	3.003	0.772	3.881	1.292	AA598776
Proliferating cell nuclear antigen	Increases processivity of leading DNA strand in replication; involved with DNA repair	PCNA	0.011	2.437	1.842	1.884	0.773	AA450265
Cyclin G1	Growth inhibitory activity linked to ARF-p53 and pRb suppressor pathways	CCNG1	0.012	2.981	0.615	2.646	0.887	AA082943
Ribonucleotide reductase M1 polypeptide	Production of deoxyribonucleotides prior to DNA synthesis in S phase of dividing cells,	RRM1	0.012	6.570	1.629	6.242	0.950	AA633549
Cell division cycle 2-like 5 (cholinesterase-related cell division controller)	Exact function unknown; family is master switch for cell cycle control	CDC2L5	0.013	0.599	0.324	0.627	1.046	AA917769
Cyclin F	Phosphorylation dependent ubiquination	CCNF	0.042	0.272	0.594	0.268	0.985	AA676797
Peanut (Drosophila)-like 1	Important for cytokinesis	PNUTL1	0.007	0.636	0.745	0.532	0.836	N92319

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold ?	Normalized values Aff	Carr	Nor	GenBank
Both								
Cell division cycle 42	General functions include cell morphology, migration, endocytosis & cell cycle, with WAS protein regulates actin which subsequently activates Arp2/3 complex;	CDC42	0.002		5.306	3.315	0.470	AA668681
M phase phosphoprotein 10	Part of U3 SnRNP in rRNA processing	MPHOSPH10	0.004		3.635	3.253	0.413	AA504113
Mitotic spindle coiled-coil related protein	Functional and dynamic regulation of mitotic spindles	SPAG5	0.026		4.566	2.711	0.624	T97349
Cell signaling								
Affected								
Epidermal growth factor	Positive regulation of cell proliferation; regulates differentiation of multiple cells types	EGF	0.026	3.575	1.639	0.604	0.458	AI480081
Ras-related C3 botulinum toxin substrate 3	Diverse functions in most cell types	RAC3	0.05	7.173	4.286	1.485	0.598	N51095
Protein kinase C, eta	May transmit EGF response, late differentiation, overexpression leads to increased TGM1	PRKCH	0.01	2.146	1.786	1.470	0.832	AA047803
Protein tyrosine phosphatase, receptor-type, zeta polypeptide 1	Role in oligodendrocyte survival and in recovery from demyelinating disease	PTPRZ1	0.041	14.459	8.241	2.529	0.570	AA476461
Protein tyrosine phosphatase, non-receptor type 12	Growth, cell differentiation, works with adhesion molecules	PTPN12	0.008	8.031	9.975	3.776	1.242	AA446259
G-protein coupled receptor 88	Neural	GPR88	0.014	8.736	4.765	1.258	0.545	N48080
C-type lectin, superfamily member 6	Inflammatory and immune response	CLECSF6	0.005	3.848	1.768	0.359	0.459	AA677149
Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Requires HIV-tat-1 and its cofactor Tat SF-1	PIK3CA	0.01	23.542	14.195	2.731	0.603	W72473
Phospholipase C, delta 4	Exact function not known	PLCD4	0.001	3.252	3.351	1.102	1.031	AA047570
A kinase (PRKA) anchor protein 5	Muscle, brain	AKAP79	0.015	6.125	3.331	1.197	0.544	N63107
Kallmann syndrome 1 sequence	Involved in FGF signaling	KAL1	0.022	4.975	3.066	0.838	0.616	H17883
Fibroblast growth factor 12	Exact function not known	FGF12	0.025	2.083	1.939	1.276	0.931	H19129
Gastrin-releasing peptide	Dermis and epidermis	GRP	0.005	0.070	0.063	0.502	0.889	AA026118
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. Required to suppress apoptosis; increase is assoc with inflammatory diseases	NFKB1	0.026	0.312	0.158	0.705	0.505	AA451716
Nuclear receptor subfamily 2, group F, member 6	Steroid hormone receptor activity	NR2F6	0.006	0.588	0.533	0.580	0.906	AA666180
Dual specificity phosphatase 8	Negatively regulates MAP kinases	DUSP8	0.014	0.080	0.058	0.214	0.723	H97140
Dual specificity phosphatase 6	Negatively regulates MAP kinases	DUSP6	0.037	0.550	0.544	0.525	0.988	AA630374
Carrier								
Endothelin receptor type A	Melanocytes	EDNRA	0.009	2.252	1.422	1.980	0.879	AA450009
PPAR binding protein	Regulates p53-dependent apoptosis, essential for adipogenesis	PPARBP	0.01	2.974	2.165	2.584	0.869	AA453404

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold ?	Normalized values Aff	Carr	Nor	GenBank
Calcitonin receptor-like	Binds adrenomedullin	CALCRL	0.006	6.972	6.891	9.797	1.405	AA757351
Protein tyrosine kinase 9	May bind actin	PTK9	0.002	7.059	3.094	5.445	0.771	AA019459
Protein tyrosine kinase 2 (focal adhesion kinase 1)	Works with fyn in epidermal differentiation	PTK2	0.049	2.289	0.328	1.608	0.703	AA482128
Tousled-like kinase 1	Nuclear serine/threonine kinase	TLK1	0.005	3.856	0.953	2.086	0.541	AA113347
Tousled-like kinase 2 (pKU-alpha protein kinase)	Nuclear serine/threonine kinase	TLK2	0.049	2.289	0.328	1.608	0.703	AA482128
Triple functional domain (PTPRF interacting)	Exact function not known	TRIO	0.01	2.684	0.455	1.472	0.548	AA007299
Serine/threonine kinase 3 (Ste20, yeast homolog)	No specific function in skin	STK3	0.003	6.884	9.019	14.297	2.077	AA464529
Fibroblast growth factor 2 (basic)	Limb and nervous system development, wound healing, tumor growth	FGF2	0.032	2.058	3.930	2.894	1.406	R38539
Phosphodiesterase 1A, calmodulin-dependent	Permanently activated in human spermatozoa	PDE1A	0.027	5.577	0.631	1.667	0.299	AA400893
TNF receptor-associated factor 4	Negatively regulates NTR induced cell death and NF-KB activation, also involved in oxidative activation of MAPK8/JNK	TRAF4	0.044	5.927	1.767	2.679	0.452	AA598826
G protein-coupled receptor 37 (endothelin receptor type B-like)	Suppresses cell death, forms a complex with Hsp70	GPR37	0.011	4.702	1.430	2.333	0.496	R66426
Neurotrophic tyrosine kinase, receptor, type 3	Mediates cell survival	NTRK3	0.019	2.509	0.799	1.987	0.792	AA774941
Src family associated phosphoprotein 2	Implicated in myeloid differentiation and growth arrest	SKAP2	0.024	4.472	3.842	5.904	1.320	R01170
Casein kinase 2, alpha prime polypeptide	Enhances beta-catenin degradation	CSNK2A2	0.005	3.547	1.369	3.285	0.926	AA054996
Nuclear receptor subfamily 6, group A, member 1	Neurogenesis and germ cell development	NR6A1	0.031	3.700	2.209	5.196	1.404	AA853954
Fibroblast growth factor 7 (keratinocyte growth factor)	Secreted by fibroblasts	FGF7	0.021	5.664	4.075	7.646	1.350	AA009609
Augmenter of liver regeneration (ERV1-homolog)	Growth factor, induces cell proliferation, particularly in liver	GFER	0.031	0.619	0.276	0.604	0.975	AA455303
Dual specificity phosphatase 16	Negatively regulates MAP kinases	DUSP16	0.048	0.492	0.553	0.408	0.828	H00288
Guanylate cyclase 1, soluble, beta 3	Functions as the main receptor for nitric oxide and nitro vasodilator drugs	GUCY1B3	0.012	0.514	0.354	0.540	1.052	AA458785
Protein phosphatase 2, regulatory subunit B (B56), gamma isoform	Negative control of cell growth and division	PPP2R5C	0.018	0.418	0.780	0.386	0.923	W35378
Chemokine (C-X-C motif), receptor 4 (fusin)	Numerous functions, important in angiogenesis	CXCR4	0.004	0.434	0.643	0.431	0.994	T62636
Protein tyrosine phosphatase, receptor type, T	Likely has roles in both signal transduction and cellular adhesion in the central nervous system	PTPRT	0.007	0.278	1.985	0.291	1.044	R52794
Guanine nucleotide binding protein (G protein), gamma 5	Signal transduction, exact function not known	GNG5	0.033	0.266	0.701	0.231	0.868	W35203
Both								
RYK receptor-like tyrosine kinase	Catalytically inactive receptor tyrosine kinase, associates with EphB2 and EphB3	RYK	0.023		2.133	2.280	0.470	T77811
Growth factor receptor-bound protein 14	Exact function not known	GRB14	0.013		3.587	4.482	0.452	R24266
Protein tyrosine phosphatase, receptor type, K	Stimulated by TGF-ß 1 which is important for the inhibition of keratinocyte proliferation	PTPRK	0.002		6.453	13.342	1.741	R79082
Protein kinase C, mu	Negative modulator of JNK signaling pathway	PRKCM	0.009		2.155	1.728	0.819	N53380

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold ?	Normalized values Aff	Carr	Nor	GenBank
Endothelin 3	Vasoactive peptides, essential for development of melanocytes	EDN3	0.037		2.846	2.292	0.808	T67004
CD47 antigen	Involved in the increase in intracellular calcium occurring upon cell adhesion to ECM, also plays a role in signal transduction	CD47	4E-04		3.528	1.759	0.352	AA455448
Transforming growth factor, alpha	Epidermal tissue regeneration, 40% sequence homology with EGF, competes with EGF for binding to EGF-R thus producing a mitogenic response	TGFA	0.034		0.137	0.404	1.032	AA933077
Cytoskeleton, motility								
Affected								
Keratin 1	Structural scaffold; expressed in suprabasal epidermis	KRT1	0.023	2.053	2.245	1.499	1.094	AA706022
Actin related protein 2/3 complex, subunit 3	Control of actin polymerization	ARPC3	0.01	2.083	2.318	2.079	1.113	H73961
Spectrin repeat containing, nuclear envelope 1	Associates with F-actin cytoskeleton and nuclear membrane	SYNE1	0.021	4.441	2.236	2.304	0.504	AA046724
Sperm specific antigen 2	Involved in the regulation of filamentous actin and signals from the outside of the cells	SSFA2	0.041	2.532	2.465	1.668	0.974	AA496804
Thymosin, beta, identified in neuroblastoma cells	Associated with actin binding, exact function not known	TMSNB	0.04	3.585	1.652	1.300	0.461	N91887
Thymosin, beta 4, X chromosome	Actin sequestering protein important in regulation of actin polymerization, also proliferation, migration, and differentiation	TMSB4X	0.039	2.087	2.261	1.873	1.084	AA634103
Flightless I homolog (Drosophila)	Cytoskeletal rearrangements	FLII	0.007	0.055	0.053	1.054	0.968	AA521453
Cysteine-rich protein 2	No exact function known	CRIP2	0.008	0.578	0.553	0.711	0.956	AA485427
Tropomyosin 2 (beta)	Muscle	TPM2	0.013	0.264	0.217	0.863	0.821	AA477400
Keratin 7	Structural scaffold; expressed in simple epithelia	KRT7	0.015	0.665	0.656	0.526	0.987	AA485959
Keratin 18	Structural scaffold; expressed in simple epithelia	KRT18	0.012	0.408	0.400	0.599	0.980	AA664179
Carrier								
Actin related protein 2/3 complex, subunit 1A	Likely controls actin polymerization in cells	ARPC1B	0.018	3.895	0.931	1.440	0.370	AA188179
PDZ and LIM domain 3	Exact function not known	PDLIM3	0.003	6.170	0.479	2.486	0.403	AA972352
Sarcoglycan, delta	Part of a complex that forms a link between F-actin cytoskeleton and the extracellular matrix	SGCD	0.001	3.227	1.357	1.844	0.571	AA234982
Neural precursor cell expressed, developmentally down-regulated 5	Involved with cytokinesis, known to associate with actin-based structures such as the contractile ring and stress fibers	NEDD5	0.038	5.210	1.446	4.460	0.856	AI025015
Wiskott-Aldrich syndrome	Dendritic cells	WAS	0.009	0.596	0.737	0.541	0.908	H61193
Peripherin	Neuronal only (receptive endings of neurons)	PRPH	0.03	0.511	0.227	0.502	0.983	AA975388
Both								
Kinesin family member 14	Exact function not known	KIF14	0.003		4.820	3.470	0.575	AA477501

Table 7. Continued

Gene description	Function	Gene name	Normalized values p-val	Fold ?	Aff	Carr	Nor	GenBank
DNA repair								
Affected								
X-ray repair complementing defective repair in Chinese hamster cells 4	Repair of DNA double-strand breaks	XRCC4	0.001	8.157	4.887	1.888	0.599	R39148
Polymerase (DNA directed), beta	DNA maintenance, replication, recombination and drug resistance	POLB	0.017	2.098	2.134	1.107	1.017	AA402855
Ectodermal-neural cortex	p53 induced gene, encodes an actin binding protein	END1	0.01	0.104	0.100	0.302	0.959	R85090
IMP (inosine monophosphate) dehydrogenase 1	Exact function unknown	IMPDH1	0.012	0.076	0.074	0.835	0.985	R52542
Carrier								
HIV TAT specific factor 1	Works with PIK3Ca	Tat-SF1	0.009	2.029	0.513	1.869	0.921	AA857131
Adenylosuccinate synthetase	Catalyzes the first step from IMP to AMP in purine synthesis	ADSS	0.004	7.611	1.944	6.031	0.792	AA431414
Single-stranded DNA-binding protein 1	Mitochondrial biogenesis	SSB	0.005	3.512	1.587	1.848	0.526	R05694
X-ray repair complementing defective repair in Chinese hamster cells 5	Repair of DNA double-strand breaks	XRCC5	0.014	9.328	1.800	3.561	0.382	AA775355
Nucleosome assembly protein 1-like 3	Exact function not determined	NAP1L3	0.003	5.032	1.477	4.445	0.883	AA463251
Translin-associated factor X	Necessary for normal proliferation	TSNAX	0.034	6.423	2.295	2.167	0.337	AA477514
Poly (ADP-ribose) glycohydrolase	Major enzyme responsible for the catabolism of poly (ADP-ribose), a reversible covalent modifier of chromosomal proteins	PARG	0.034	0.562	0.947	0.548	0.976	H95088
Histone deacetylase 1	Deacetylates p53, promotes cell growth and decreased apoptosis	HDAC1	0.013	0.416	0.983	0.416	1.001	AA465353
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	DNA repair	NUDT1	0.019	0.599	0.688	0.547	0.914	AA443998
Both								
Polymerase (DNA directed), gamma 2, accessory subunit	Mitochondrial DNA replication	POLG	0.021		5.970	3.961	0.801	AI023804
Histone acetyltransferase 1	Exact function not known	MYST1	0.007		5.381	3.718	0.502	AA625662
Endocrine								
Affected								
Hydroxysteroid (11-beta) dehydrogenase 2	Required for selective expression of aldosterone action on the mineralocorticoid receptor	HSD11B2	0.02	2.085	2.124	1.870	1.018	W95082
Adrenomedullin	Enhances keratinocyte growth by stimulating proliferation and inhibiting apoptosis	ADM	0.039	0.415	0.162	0.597	0.390	AA446120
Sulfonylurea receptor	Hyperplastic skin reactions have occurred as a side effect of sulfonylurea preparations	SUR1	0.028	0.253	0.228	0.667	0.901	AI292126
Corticotropin releasing hormone receptor 1	May be involved in the stress response in skin; suppresses keratinocyte proliferation	CRHR1	0.012	0.156	0.069	0.669	0.442	H07089
Insulin-like growth factor 1 (somatomedin C)	Important mediator of keratinocyte growth in vitro, may also regulate growth in vivo; pattern of expression distinct from EGF	ILGF1	0.047	0.243	0.182	0.502	0.750	AA456321

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold ?	Normalized values Aff	Carr	Nor	GenBank
Carrier								
Prolactin receptor gene	Has been demonstrate in hair follicles		0.006	7.179	3.899	16.819	2.343	AA704894
Progesterone receptor membrane component 2	Function not yet determined	PGRMC2	0.033	0.439	0.396	0.257	0.586	AA047567
Insulin-like growth factor binding protein 5	Enhanced keratinocyte cell migration and proliferation such as in wound healing and skin regeneration	IGFBP5	0.008	0.500	0.757	0.473	0.946	H08561
Both								
Mineralocorticoid receptor (aldosterone receptor)	Requires HSD11B2 for mineralocorticoid specific binding, expressed in epidermis but exact function not yet known	NR3C2	0.034		6.257	5.224	0.640	AA447079
Extracellular matrix								
Affected								
Collagen, type XV, alpha 1	Wide tissue distribution, strongest expression in basement membrane so may help adhere to underlying connective tissue	COL15A1	0.001	3.454	3.139	1.282	0.909	AA455157
Collagen, type I, alpha 2	Type I, fibrillar, collagen found in most connective tissues	COL1A2	0.021	0.581	0.616	1.384	1.060	AA490172
Collagen, type IX, alpha 3	Major collagen component of hyaline cartilage, usually found in tissues containing type II collagen, a fibrillar collagen	COL9A3	0.041	0.467	0.433	0.723	0.927	AA017526
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	Oxidoreductase activity, can be used to selectively alter glycosylation and hydroxylation	PLOD3	0.011	0.357	0.376	0.734	1.053	AA905976
Collagen, type 6, alpha 1	Major structural component of microfibrils	COL6A1	0.048	0.503	0.456	0.959	0.906	AA046525
Matrix metalloproteinase 17 (membrane-inserted)	Unique MMP that is GPI anchored, activates MMP2	MMP17	0.004	0.561	0.527	1.168	0.940	R42600
Carrier								
Fibrillin 1	Component of extracellular microfibrils, found in elastic and nonelastic connective tissues of the body	FBN1	0.016	8.054	0.393	3.969	0.493	AA418674
Syndecan 2	Role in mediating adhesion and proliferation	SDC2	0.002	3.747	0.726	2.023	0.540	H64347
Matrilin 2	Family of proteins involved in formation of filamentous networks in ECM of many tissues, specific function not yet determined	MATN2	0.004	6.914	9.134	16.646	2.408	AA071473
Laminin, alpha 5	Major component of basement membranes	LAMA5	0.013	2.181	0.267	1.756	0.805	AA459289
Procollagen-proline, 2-oxoglutarate 4-dioxygenase, beta polypeptide	Exact function not known	P4HB	0.049	2.595	1.105	2.339	0.901	R27004
Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha polypeptide 1	Oxidoreductase activity, exact function not known	P4HA1	0.027	2.086	1.081	1.801	0.863	AA457671
EGF-containing fibulin-like extracellular matrix protein 1	Exact function not known	EFEMP1	0.019	6.912	1.243	4.728	0.684	AA875933
Tubulin tyrosine ligase	Posttranslational modification of alpha-tubulin; participates in a cycle of tubulin detyrosination and tyrosination	TTL	0.001	4.732	1.234	4.780	1.010	AA682816
Matrix metalloproteinase 1	Breaks down the interstitial collagens, types I, II, and III	MMP1	0.015	2.067	0.359	1.554	0.752	AA143331
Heparan sulfate proteoglycan	Major component of basement membranes, associated with surface of fibroblasts	HSPG2	0.002	0.577	0.538	0.548	0.949	AA427561

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Both								
Laminin, alpha 4	important role in cell adhesion and/or vessel wall formation in the skin by interacting with syndecan-2 and/or -4	LAMA4	0.006		6.820	11.832	1.011	R43734
RBC-related								
Affected								
Coagulation factor VIIIc, procoagulant component	Coagulation	F8	0.049	10.579	4.892	1.537	0.462	AA426469
Phosphofructokinase, platelet	Regulatory enzyme of glycolysis, no specific function for platelet isoform given	PFKP	0.007	0.213	0.156	2.513	0.732	AA608558
Hemoglobin, zeta	Synthesized in the yolk sac of the early embryo	HBZ	0.022	0.460	0.441	0.721	0.959	N59636
Plasminogen activator, urokinase receptor	Activated in stratum corneum after barrier disruption, converts plasminogen into plasmin which can cleave many proteins, including ECM proteins and MMP precursors	PLAUR	0.007	0.139	0.094	1.067	0.672	AA454879
Carrier								
Protein C inhibitor (plasminogen activator inhibitor III)	Protease inhibitory activity, constitutively expressed by keratinocytes in culture, may regulate retinoid supply	PCI	0.011	5.232	1.019	2.343	0.448	AA858026
Coagulation factor XIII, A1 polypeptide	Links fibrin monomers with collage, protein is expressed in epidermis and on dermal dendritic cells, specific function unknown	F13A1	0.014	6.212	2.123	3.530	0.568	AA449742
Small membrane protein 1	Exact function not known	SMP1	0.039	2.880	1.735	1.914	0.665	H07132
Pyruvate kinase, liver and RBC	Production of phohsphoenolpyruvate from pyruvate and ATP, associated with nonspherocytic hemolytic anemia	PKLR	0.018	0.574	0.634	0.516	0.898	R08829
Hemoglobin, epsilon 1	Normally expressed in the embryonic yolk sac	HBE1	0.001	0.203	0.051	0.151	0.743	H79534
Biliverdin reductase B	Exact function not known	BLVRB	0.037	0.553	0.228	0.519	0.939	AA857035
Solute carrier family 14, member 1	Encodes the Kidd blood group antigens	SLC14A1	0.05	0.541	0.237	0.418	0.771	H82236
Protein C inhibitor (plasminogen activator inhibitor III)	Regulation of retinoid supply in the epidermis, constitutively expressed by keratinocytes in culture	SERPINA5	0.027	0.640	0.315	0.663	1.035	W86431
Glycophorin A (includes MN blood group)	Major sialoglycoprotein of human RBC membrane	GYPA	0.034	0.525	0.469	0.516	0.983	AA455338
Both								
Solute carrier family 4, anion exchanger, member 1 (Band 3 of RBC)	Found on RBC plasma membrane, functions as a chloride and bicarbonate exchanger for CO2 transport from tissues to lungs	SLC4A1	0.014		4.021	5.971	1.070	T86708
Hepatic metabolism								
Affected								
Catechol-O-methyltransferase	Major degradative pathways of the catecholamine transmitters	COMT	0.008	3.041	3.029	1.626	0.996	R44202
Cytochrome c oxidase subunit VIc	Terminal enzyme of the mitochondrial respiratory chain, catalyzes the electron transfer from cytochrome c to oxygen	COX6C	0.044	2.005	2.019	1.376	1.007	AA456931
Sulfotransferase family 1C, member 1	Generally, catalyzes sulfate conjugation hormones, neurotransmitters, drugs, and xenobiotic compounds	SULT1C1	0.018	5.475	2.186	1.668	0.399	W88655

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Cytochrome c oxidase subunit VII-related protein	Expressed in all tissues, may mediate the higher level of energy production in target cells by estrogen	COX7A2L	0.031	0.479	0.289	0.817	0.603	R10896
Carrier								
Cytochrome P450, family 19, subfamily A, polypeptide 1	Catalyzes the last step of estrogen synthesis	CYP19A1	0.002	9.155	1.682	3.851	0.421	AA628233
Cysteine dioxygenase, type I	Exact function not known	CDO1	0.023	6.620	9.020	16.040	2.423	AA497033
Cytochrome b5 outer mitochondrial membrane precursor	Exact function not known	CYB5-M	0.03	0.618	0.753	0.588	0.952	W04674
Guanidinoacetate N-methyltransferase	Converts guanidoacetate to creatine, may be involved with neurologic syndromes and muscular hypotonia	GAMT	0.01	0.636	0.676	0.637	1.003	AA521337
Both								
Ubiquinol cytochrome c reductase core protein II	Exact function not known	UQCRC2	0.002		6.915	8.158	1.266	R12802
Advanced glycosylation end product-specific receptor	Immunoglobulin superfamily, receptor for various molecules, inc the serum amyloid A and S100/calgranulin superfamily	AGER	0.033	0.649	0.794	0.606	0.934	W74536
Major histocompatibility complex, class II, DR beta 1		HLA-DRB1	0.046	0.577	0.939	0.591	1.024	AA664195
Interferon (alpha, beta and omega) receptor 1		INFAR1	0.032	0.217	0.415	0.174	0.800	N59150
Both								
CD47 antigen		CD47	4E-04		3.528	1.759	0.352	AA455448
Intracellular trafficking								
Affected								
Nucleobindin 2	Exact function no known	NUCB2	0.001	2.374	2.103	0.966	0.886	AA485214
Early endosome antigen 1, 162kD	Associated with maturation of phagolysosomes	EEA1	0.002	10.150	4.648	1.890	0.458	N66043
Syntaxin 7	Regulates membrane transport with late endosomes and lysosomes, associated with intracellular vacuolation	STX7	0.04	3.402	1.893	0.754	0.556	T71551
Solute carrier family 22 (organic cation transporter) member 4	Organic cation transporter, critical for elimination of endogenous small organic cations and many drugs and environmental toxins	SLC22A4	0.049	0.476	0.383	1.093	0.806	N26836
Metallothionein IF	Upregulated by HIV-1 tat in immature dendritic cells, exact function not known	MT1F	0.046	0.314	0.234	0.687	0.745	N55459
Kinesin family member 1A	Transports membranous organelles along axonal microtubules	KIF1A	0.025	0.185	0.100	1.345	0.538	H92234
Carrier								
BET1 homolog	Golgi-associated protein that participates in vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus	BET1	0.003	8.796	3.759	15.189	1.727	H54289
Coatomer protein complex, subunit beta 2	Exact function not known	COPB	0.018	3.004	1.287	3.123	1.040	AA598868
Steroidogenic acute regulatory protein	Transport of cholesterol to inner mitochondrial membrane, where first step of steroidogenesis takes place	STAR	0.017	4.963	1.087	2.295	0.463	AA679454
Sorting nexin 2	Resides primarily in early endosomes, regulates lysosomal sorting of internalized EGFR	SNX2	0	2.097	1.995	1.811	0.864	AA169814

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Spastic paraplegia 20	Likely involved in endosomal trafficking and/or microtubule dynamics	SPG20	0.038	4.810	1.884	2.148	0.447	AA953973
ATP-binding cassette, sub-family C (CFTR/MRP), member 5	Provides resistance to thiopurine anticancer drugs	ABCC5	0.004	2.823	1.089	1.580	0.560	N68159
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	Sorting receptor for retention of resident soluble proteins in the lumen of the endoplasmic reticulum	KDELR2	0.002	5.837	3.215	2.018	0.346	AA626867
Transportin 2	mRNA export from the nucleus	TNPO2	0.005	0.577	0.643	0.504	0.874	AA778206
Both								
Secretory carrier membrane protein 1	Carriers to the cell surface in post-golgi recycling pathways	SCAMP1	0.035		39.478	4.662	1.199	AA490945
Ion channel, membrane potential								
Affected								
Mercurial-insensitive water channel, form 2	Water channel present on keratinocytes	AQP4	0.011	10.905	13.770	8.440	1.263	R41928
ATPase, Na+/K+ transporting, alpha 1 polypeptide	Integral membrane protein for establishing & maintaining electrochemical gradients of Na and K ions across the plasma membrane	ATP1A1	0.05	2.637	2.839	1.754	1.077	AA873355
Potassium large conductance calcium-activated channel, subfamily M, beta member 1	Fundamental to the control of smooth muscle tone and neuronal excitability	KCNMB1	0.048	0.273	0.111	1.458	0.408	AA029299
Annexin A11	Assoc with mitotic spindles and may have role in cell cycle; Ca-dependent phospholipid binding protein; implicated in exocytotic and endocytotic pathways	ANXA11	0.002	0.234	0.148	0.887	0.635	AA464982
Carrier								
Annexin A4	Almost exclusively expressed in epithelial cells; Ca-dependent phospholipid binding protein; implicated in exocytotic and endocytotic pathways	ANXA4	2E-04	5.260	0.602	3.448	0.656	AA419015
Claudin 16	Integral membrane protein and component of tight junctions	CLDN16	0.036	4.846	1.202	2.570	0.530	AA777384
Potassium channel tetramerisation domain containing 10	Exact function not known	KCTD10	0.014	2.059	2.075	2.364	1.148	H16796
ATPase, Ca++ transporting, plasma membrane 4	Intracellular calcium homeostasis	ATP2B4	0.006	4.332	1.159	2.100	0.485	N93024
Potassium inwardly-rectifying channel, subfamily J, member 15	Integral membrane protein & potassium channel, has a greater tendency to let potassium to flow into rather than out of a cell	KCNJ15	0.039	0.444	1.052	0.414	0.934	T94781
Both								
Potassium voltage-gated channel, shaker-related subfamily, beta member 1,	Forms a complex with alpha subunits and modulate the activity of the pore-forming alpha subunits	KCNAB1	0.001		33.233	11.147	1.806	AA013095
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	Exact function not known	ATP5F1	0.048		2.226	2.058	1.028	AA453765

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Protein and amino acid metabolism								
Affected								
Arginase, type II	Located in mitochondria, catalyzes the hydrolysis of arginine to ornithine and urea	ARG2	0.019	6.646	5.073	1.672	0.763	H17612
Pepsinogen A	Diagnostic marker of gastric disorders	PGA	0.003	0.452	0.492	0.669	1.087	R72097
Alpha-2-glycoprotein 1, zinc	Exact function not known	AZGP1	0.043	0.148	0.104	0.614	0.700	AA677165
Cathepsin D (lysosomal aspartyl protease)	Lysosomal aspartyl protease, first expressed as proenzyme in spinous layer, activated in the lysosomes in the granular layer	CTSD	0.005	0.604	0.544	1.028	0.900	N20475
Carrier								
Glycine cleavage system protein H	Mitochondrial enzyme, cleavage of glycine	GCSH	0.03	7.209	0.969	2.831	0.393	R28294
Isovaleryl Coenzyme A dehydrogenase	Mitochondrial enzyme, catalyzes 3rd step in leucine catabolism	IVD	0.049	4.371	0.582	2.158	0.494	AA464149
3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	Mitochondrial enzyme, amino acid metabolism	HMGCL	0.049	0.651	0.901	0.661	1.015	AA458779
Both								
Ornithine decarboxylase 1	Rate-limiting enzyme of the polyamine biosynthesis pathway which catalyzes ornithine to putrescine	ODC1	1E-04		3.082	2.373	0.855	AA461467
Tripeptidyl peptidase II	Exact function not known	TPP2	0.021		2.519	1.983	0.651	R39682
Presenilin enhancer 2	Required for Notch pathway signaling, and for the activity and accumu lation of gamma-secretase	PEN2	0.007		2.346	2.361	0.965	AA435940
Glycoprotein 2 (zymogen granule membrane)	Exact function not known	GP2	0.017		0.404	0.359	0.899	AA844930
Protein folding and degradation								
Affected								
Proteasome subunit, alpha type, 5	Part of 20S subunit, TIA family, cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway	PSMA5	0.047	3.380	2.471	1.207	0.731	AA598815
Proteasome subunit, beta type, 7	Part of 20S subunit, TIB family, cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway	PSMB7	0.027	2.286	2.641	1.386	1.155	AA489343
Ubiquitin protein ligase E3C	Exact function not known	UBE3C	0.004	14.347	19.673	1.741	1.371	AA284827
SUMO1/sentrin specific protease 1	Mediates SUMO-1, a protein modifier	SENP1	0.025	12.349	19.845	3.158	1.607	AA489050
Peroxisomal biogenesis factor 12	Exact function not known	PEX12	0.007	4.600	1.807	0.574	0.393	H10965
Proteasome subunit, beta type, 1	Part of 20S subunit, TIB family, cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway	PSMB1	0.007	2.474	2.563	1.395	1.036	T68758
Proteasome 26S subunit, ATPase, 2	ATPase subunit with chaperone-like may also regulate transcription activity	PSMC2	0.034	2.880	2.675	1.497	0.929	AA251770
Proteasome 26S subunit, ATPase, 6	ATPase subunit with chaperone-like may also regulate transcription activity	PSMC6	0.031	3.210	3.230	2.087	1.006	AA424503
Ubiquitin-conjugating enzyme E2 variant 2	Associated with signaling cellular stress	UBE2V2	0.02	3.312	3.753	2.543	1.133	AA448676
Proteasome 26S subunit, ATPase, 3	ATPase subunit with chaperone-like may also regulate transcription activity	PSMC3	0.015	0.523	0.466	0.608	0.892	AA282230

Table 7. Continued

Gene description	Function	Gene name	Normalized values p-val	Fold?	Aff	Carr	Nor	GenBank
Carrier								
Proteasome 26S subunit, non-ATPase 3	ATPase subunit with chaperone-like may also regulate transcription activity	PSMD3	0.021	2.048	1.722	1.509	0.737	AA485052
SUMO1/sentrin/SMT3 specific protease 2	Mediates SUMO-2, a protein modifier	SENP2	0.016	4.872	0.589	2.296	0.471	AA705377
Ubiquitin specific protease 9, X chromosome	May regulate protein levels by removing ubiquitin from proteins marked for digestion by proteasomes	USP9X	0.034	8.392	1.159	2.762	0.329	AA425628
Ubiquitin-conjugating enzyme E2B	Post-replicative DNA damage repair	UBE2B	0.021	2.297	2.031	1.875	0.816	AA598492
Ubiquitin-activating enzyme E1	First step in ubiquitin conjugation to mark cellular proteins for degradation, may function in DNA repair	UBE1	0.009	3.489	0.953	3.003	0.860	AA598670
Chaperonin containing TCP1, subunit 6A (zeta 1)	Exact function not known	CCT6A	0.002	6.844	2.837	7.139	1.043	AA872690
Ubiquitin-activating enzyme E1	First step in ubiquitin conjugation to mark cellular proteins for degradation, may function in DNA repair	UBE1	1E-04	7.059	2.067	3.409	0.483	R61332
Ubiquitin-like 4	Exact function not known	UBL4	0.012	0.307	0.305	0.358	1.166	AA485397
Nuclear VCP-like	Exact function not known	NVL	0.011	0.503	0.577	0.478	0.950	W86860
Ubiquitin-conjugating enzyme E2A	Post-replicative DNA damage repair	UBE2A	0.01	0.629	0.924	0.660	1.048	AA600173
Peptidylprolyl isomerase F (cyclophilin F)	Accelerates protein folding	PPIF	0.05	0.576	1.015	0.473	0.821	AA404286
Both								
Proteasome regulatory particle subunit p44S10	Exact function not known	P44S10	0.005		3.630	2.469	0.835	AA424807
Cytoplasmic chaperonin hTRiC5	Chaperone activity, exact function not known	CCT3	0.011		2.450	2.224	1.090	R60933
Translation								
Affected								
Aspartyl-tRNA synthetase	Charges cognate tRNA with aspartate during protein synthesis	DARS	0.041	2.119	1.679	0.624	0.792	AA481562
Alanyl-tRNA synthetase	Charges cognate tRNA with alanine during protein synthesis	AARS	0.046	4.317	1.800	1.088	0.417	AA156571
Eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	Early steps of protein synthesis, forms complex with GTP, initiator tRNA and binds to a 40S ribosomal subunit	EIF2S2	0.026	2.062	2.298	1.417	1.115	AA027240
Eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	Early steps of protein synthesis, forms complex with GTP, initiator tRNA and binds to a 40S ribosomal subunit	EIF2S2	0.033	2.319	2.296	1.490	0.990	R54097
Ribosomal protein S8	Catalyze protein synthesis	RBS8	0.028	2.112	2.202	0.900	1.043	AA683050
Ribosomal protein L11	Catalyze protein synthesis	RBL11	0.025	2.415	2.887	2.072	1.195	AA680244
Ribosomal protein L5	Catalyze protein synthesis	RBL5	0.037	2.112	2.295	1.770	1.087	AA496880
Ribosomal protein L36a	Catalyze protein synthesis	RBL36A	0.038	2.225	2.458	1.873	1.105	AA669359
Eukaryotic translation initiation factor 2B, subunit 3, gamma	Exact function not known	EIF2B3	0.046	0.247	0.179	0.428	0.723	W58368
Carrier								
Eukaryotic translation elongation factor 1 alpha 1	Responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome	EEF1A1	0.004	5.481	1.979	3.271	0.597	W32408
Isoleucine-tRNA synthetase	Link amino acids with nucleotide triplets	IARS	0.012	5.821	1.316	2.237	0.384	AA410636

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Eukaryotic translation elongation factor 1 epsilon 1	Exact function not known	EEF1E1	0.004	22.983	8.284	24.028	1.045	N91962
Mitochondrial translational initiation factor 2	Promotes the binding of the initiator tRNA to the small subunit of the ribosome in a GTP-dependent manner	MTIF2	0.011	4.143	0.700	1.983	0.479	H18070
Fibrillarin	Component of a snRNP particle thought to participate in the first step in processing preribosomal RNA	FBL	0.027	0.578	0.745	0.509	0.880	AA663986
Lipid metabolism								
Affected								
Acyloxyacyl hydrolase (neutrophil)	May modulate host inflammatory responses to gram-negative bacteria	AOAH	0.026	3.982	2.517	1.932	0.632	T65864
Alpha-1-antichymotrypsin	Produced by mast cells	SERPINA3	0.026	7.930	3.464	0.975	0.437	AA703191
Apolipoprotein B (including Ag(x) antigen)	Increased expression with increased layers in spinous and granular layers	APOB	0.017	4.329	3.735	0.298	0.863	H93332
Mitochondrial short-chain enoyl-CoA hydratase	Functions in the second step of the mitochondrial fatty acid beta-oxidation pathway	ECHS1	0.033	3.576	1.837	1.160	0.514	R43558
Fatty acid binding protein 4, adipocyte	Epidermis expresses abundant FABPs but this gene has no specific function listed for epidermis	FABP4	0.008	2.470	2.759	0.704	1.117	N92901
Fatty acid binding protein 7, brain	Epidermis expresses abundant FABPs but this gene has no specific function listed for epidermis	FABP7	0.006	6.250	2.583	1.211	0.413	W72051
Steroid sulfatase (microsomal)	Concentrated in lamellar bodies, generates cholesterol for barrier formation	STS	0.018	0.522	0.419	0.521	0.803	H15155
Sulfotransferase family 2B, member 1	2 isoforms, one isoform is associated with epidermal differentiation	SULT2B1	0.027	0.302	0.328	0.539	1.086	R73584
Arachidonate 5-lipoxygenase	Present in human epidermal Langerhans cells	ALOX5	0.032	0.386	0.289	1.420	0.747	H51574
Phosphodiesterase 6G, cGMP-specific, rod, gamma	Works with EGF	PDE6G	0.035	0.607	0.626	0.616	1.031	AA074148
Carrier								
Glycosylphosphatidylinositol specific phospholipase D1	GPI degrading enzyme	GPLD1	0.041	3.223	0.837	1.804	0.560	N69672
Acid phosphatase 1, soluble	Hydrolyzes protein tyrosine phosphate to protein tyrosine and orthophosphate	ACP1	0.034	0.597	1.227	0.424	0.710	W45148
Both								
Palmitoyl-protein thioesterase	Produced by fibroblasts and neural cells	PPT1	0.007		0.490	0.444	0.869	AA063637
Neutral sphingomyelinase	Reduced activity correlates with impaired expression of cornified envelope proteins and keratins	SMPD2	0.021		0.417	0.327	0.898	AA680132
Carbohydrate metabolism								
Affected								
Lactate dehydrogenase-A	Found mainly in muscle, catalyzes conversion of L-lactate & NAD to pyruvate & NADH in final step of anaerobic glycolysis	LDHA	0.028	2.097	2.137	1.252	1.019	H05914
Lactate dehydrogenase B	Exact function not known	LDHB	0.019	2.710	2.826	1.686	1.043	AA629567

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1	Mucin-type O-glycosylation	GALNT1	0.025	3.407	2.419	0.640	0.710	AA029851
Galactose-4-epimerase, UDP-	Catalyzes 2 analogous reactions: epimerization of UDP-glucose to UDP-galactose, and UDP-N-acetylglucosamine to UDP-N-acetylgalactosamine	GALE	2E-04	0.034	0.037	0.167	1.091	AA280832
Glucosamine (N-acetyl)-6-sulfatase	Lysosomal enzyme, catabolism of heparin, heparan sulfate, and keratan sulphate	GNS	0.03	0.460	0.380	0.642	0.826	AA035347
Carrier								
Mannose phosphate isomerase	Maintains supply of D-mannose derivatives, required for most glycosylation reactions	MPI	0.025	4.281	1.425	1.988	0.464	H15442
Dolichyl-phosphate N-acetylglucosaminephosphotransferase 1	Catalyzes first step in dolichol-linked oligosaccharide pathway for glycoprotein biosynthesis	DPAGT1	0.034	0.426	1.431	0.413	0.969	R55620
Both								
Aldehyde reductase 1 (low Km aldose reductase)	Catalyzes the reduction of a number of aldehydes	AKR1B1	0.003		14.266	8.801	0.875	AA701963
Energy metabolism, respiratory chain, nucleic acid synthesis								
Affected								
NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 (6kD, KFYI)	Exact function not known	NDUFV2	0.041	2.094	2.487	1.064	1.188	AA460251
Solute carrier family 25, member 5	Mitochondrial carrier; adenine nucleotide translocator	SLC25A5	0.027	2.112	2.391	1.614	1.132	AA404486
Malate dehydrogenase 1, NAD (soluble)	Pivotal roles in malate-aspartate shuttle that operates coordination between cytosol and mitochondria	MDH1	0.049	2.053	2.466	0.842	1.201	AA403295
Dihydropyrimidine dehydrogenase	Pyrimidine catabolic enzyme	DPYD	0.023	4.887	2.037	1.440	0.417	AA430625
IMP (inosine monophosphate) dehydrogenase	IMPDH1 is a ubiquitously expressed enzyme, functioning as a homotetramer, which catalyzed the rate-limiting step in de novo synthesis of guanine nucleotides	IMPDH1	0.012	0.204	0.131	0.666	0.643	AA461048
Carrier								
Malic enzyme, NADP+-dependent, mitochondrial	Catalyzes the oxidative decarboxylation of malate to pyruvate using NADP as a cofactor	ME3	0.009	5.078	0.730	1.904	0.375	AA779401
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent)	Mitochondrial bifunctional enzyme, electron transporter activity	MTHFD2	0.005	6.597	7.138	7.994	1.212	AA633577
Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	de novo synthesis of pyrimidine nucleotides, regulated by MAPK cascade	CAD	0.03	7.825	13.928	8.324	1.780	R85414
Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	de novo purine biosynthesis	GART	0.032	6.005	1.510	3.705	0.617	AA598487
Both								
Cytochrome c-1, somatic	Involved with initiation of apoptosis, a component of the electron transport chain in mitochondria	CYCS	0.018		3.811	2.257	0.520	R52654

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Oncogenes								
Affected								
RAB5A, member RAS oncogene family	Regulator of endocytosis	RAB5A	0.026	3.809	3.363	2.440	0.883	H11455
RAB32, member RAS oncogene family	Mitochondrial anchoring of PKA and synchronization of mitochondrial fission.	RAB32	0.016	5.210	3.225	1.091	0.619	AA057378
ALL1-fused gene from chromosome 1q	Restricted distribution in normal hematopoietic tissues but fused with many genes in leukemia	AF1Q	0.023	6.018	2.470	0.682	0.410	AA456008
ELK1, member of ETS oncogene family	Transcription factor activity	ELK1	0.032	4.393	4.119	1.796	0.938	AA844141
Ecotropic viral integration site 1	Promotes cell proliferation	EVI1	0.008	2.006	2.158	0.787	1.076	AA181023
Pre-B-cell leukemia transcription factor 1	Megakaryocytic gene expression	PBX1	0.006	2.889	2.622	1.758	0.908	AA403031
v-abl Abelson murine leukemia viral oncogene homolog 1	Implicated in cell differentiation, division, adhesion and stress response	ABL1	0.001	10.160	2.740	1.490	0.270	AA496785
RAB2, member RAS oncogene family	Interacts directly with atypical PKC iota/lambda and inhibits a PKC iota/lambda-dependent GADPH phosphorylation	RAB2	0.02	6.106	2.490	1.680	0.408	T82415
Estrogen receptor-binding fragment-associated gene 9	Tumor-associated antigen response to estrogen	EBAG9	0.026	18.937	17.726	1.393	0.936	T50699
SH3-domain GRB2-like 1	Interferes with Ras-suppressing activities	SH3GL1	0.004	0.613	0.625	0.826	1.019	AA398366
v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) opposite strand	Implicated in the pathogenesis of a number of tumors	MYCNOS	0.002	0.512	0.499	0.985	0.973	AA609982
Carrier								
Wingless-type MMTV integration site family member 2	Regulation of cell fate and patterning during embryogenesis	WNT2	0.049	2.068	1.367	2.133	1.032	T99055
FBJ murine osteosarcoma viral oncogene homolog B	Forms the transcription factor complex AP-1, regulates cell proliferation, differentiation, and transformation	FOSB	0.011	5.589	3.323	8.879	1.589	T61948
v-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived	Promotes survival , allows escape from growth suppression activity of p73	MYCN	0.011	2.753	1.579	2.544	0.924	R52824
v-myc avian myelocytomatosis viral oncogene homolog	Promotes survival by stimulating cell growth and decreasing apoptosis, interferes with DNA repair	MYC	0.018	0.562	0.544	0.575	1.022	AA464600
Non-metastatic cells 1, protein expressed in	Metastasis suppressor gene	NME1	0.028	0.665	1.089	0.676	1.016	AA644092
Both								
Pim-2 oncogene	Pro-survival kinase	PIM2	0.04		3.275	2.280	0.991	AA863383
Ewing sarcoma breakpoint region 1	Expressed in keratinocytes	EWSR1	0.027		1.999	2.082	0.976	AA464184
Meningioma expressed antigen 6	May cause immune reaction in meningioma/glioma patients	MGEA6	0.037		8.907	4.024	1.129	T99793
Rho guanine nucleotide exchange factor(GEF) 12	May form a complex with G proteins and stimulate Rho-dependent signals	ARHGEF12	0.008		7.879	16.165	1.887	AA479287
Transcription								
Affected								
Sp 110 nuclear body	Can function as an activator of gene transcription and may serve as a nuclear hormone receptor coactivator	SP110	2E-04	7.231	5.533	1.776	0.765	T62627

Table 7. Continued

Gene description	Function	Gene name	Normalized values p-val	Fold?	Aff	Carr	Nor	GenBank
Homeo box D4	Transcription factor, retinoic acid response factor, may play a role in determining positional values in developing limb buds	HOXD4	1E-04	3.122	2.024	0.845	0.648	AA447692
High mobility group AT-hook 2	Role in adipogenesis & mesenchymal differentiation suggested	HMGA2	0.023	16.740	15.670	1.793	0.936	H98218
Heterogeneous nuclear ribonucleoprotein A2/B1	Ubiquitously expressed, influence pre-mRNA processing and mRNA metabolism and transport	HNRPA2B1	0.016	2.367	2.684	2.030	1.134	W02101
FUS-interacting protein 1	Constitutive and regulated RNA splicing	FUSIP1	0.002	3.056	2.989	1.879	0.978	N30285
Developmentally regulated GTP-binding protein 1	Metastasis suppressor gene	DRG1	0.028	2.175	2.060	1.878	0.947	AA488336
Activating transcription factor 3	Represses transcription	ATF3	0.028	6.601	10.257	9.766	1.554	H21042
p300/CBP-associated factor	Coactivator with intrinsic histone acetyl transferase activity, associated with differentiation	PCAF	0.039	4.889	8.603	5.989	1.760	N74637
Basic transcription factor 3	Exact function not known	BTF3	0.032	2.011	2.312	1.786	1.150	R83000
RNA binding motif, single stranded interacting protein 1	Bind single-stranded DNA/RNA, implicated in multiple diverse functions	RBMS1	0.005	2.333	2.566	1.248	1.100	N31587
Forkhead box F1	Specific function not known, but may have role in embryonic development	FOXF1	0.007	2.232	2.415	2.081	1.082	AA112660
General transcription factor IIH, polypeptide 1	Inhibits HIV-1 Vpr cell cycle arresting function, initiates transcription	GTF2H1	0.022	6.741	2.780	1.210	0.412	AA455004
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide1	RNA helicase which generally functions to alter RNA secondary structure, nuclear splicing, and spliceosome assembly, exact function not known	DDX1	0.038	2.197	2.148	1.951	0.978	AA425687
Zinc finger protein 167	Exact function not known	ZNF167	0.004	10.584	6.099	2.329	0.576	R94845
U2 (RNU2) small nuclear RNA auxiliary factor 2	Non-snRNP protein required for the binding of U2 snRNP to the pre-mRNA branch site	U2AF2	0.049	0.141	0.084	0.118	0.593	AA405748
GA-binding protein transcription factor, beta subunit 2	Activation of cytochrome oxidase expression and nuclear control of mitochondrial function	GABPB2	0.001	0.203	0.116	0.583	0.571	H91651
Ring finger protein 1	Can bind DNA and act as a transcriptional repressor	RFP1	0.036	0.506	0.476	0.534	0.942	AA425772
Activating transcription factor 6	Activates the transcription of ER molecules	ATF6	0.05	0.187	0.153	0.547	0.817	AA707661
Zinc finger protein 36, C3H type-like 2	Regulates response to growth factors	ZFP26L2	0.003	0.440	0.451	0.722	1.024	AA480880
Proline rich 5 (salivary)	Exact function not known	PB1	0.028	0.135	0.087	0.659	0.648	AA447734
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	Helicase and ATPase activities, regulate transcription by altering chromatin structure	SMARCA3	0.037	0.568	0.581	0.568	1.022	H24688
Serine proteinase inhibitor, clade I, member 1	Expressed in the nervous system and is an inhibitor of tpa and plasmin	SERPINI1	0.002	0.459	0.225	1.189	0.490	AA115877
Small nuclear ribonucleoprotein polypeptide N	Pre-mRNA processing, possibly tissue-specific alternative splicing events	SNRPN	0.009	0.591	0.588	0.608	0.995	T54926
Ets2 repressor factor	Exact function not known	ERF	0.034	0.129	0.142	0.610	1.106	W86216
Homeo box B13	Expressed in fetal skin, increased expression during epidermal regeneration	HOXB13	0.006	0.059	0.045	0.637	0.759	AA456069

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Carrier								
E2F transcription factor 5, p130-binding	Expressed in a wide variety of tissues, interacts with the tumor suppressors, p130 and p107	E2F5	0.049	3.729	1.429	3.294	0.883	AA455521
Zinc finger protein, X-linked	Exact function not known	ZFX	0.011	4.229	2.811	4.057	0.959	AA406372
Transformer-2 alpha	Splicing activator that likely participates in the control of cell-cell splicing patterns	TRA2A	0.017	2.415	1.648	1.677	0.695	R09691
Runt-related transcription factor 2	Essential for regulation of osteoblast differentiation	RUNX2	0.034	3.083	0.512	1.520	0.493	AA858175
Transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C	Activates elongation by RNA polymerase II by suppressing transient pausing of the polymerase	TCEB1	1E-04	3.690	1.270	1.571	0.426	W81191
Transcription elongation regulator 1	Regulation of transcription elongation	TCERG1	0.014	2.109	1.529	1.706	0.809	AA045180
C-myc promoter-binding protein	Exact function not known	IRLB	0.013	3.225	5.992	6.414	1.989	N50544
Pirin	Iron-binding nuclear protein and transcription cofactor	PIR	0.002	3.247	2.011	1.974	0.608	H69335
Suppressor of RNA polymerase B, yeast homolog	Exact function not known	SRB7	0.006	9.944	1.390	7.302	0.734	AA130633
PBX/knotted 1 homeobox 1	Cofactor with PBX , induced by retinoic acid, neurologic and ovarian effects	PKNOX1	0.011	3.516	0.534	1.795	0.510	T96688
Splicing factor proline/glutamine rich	Multi-functional nuclear protein, pre-mRNA splicing factor activity and mRNA splicing	SFPQ	0.046	5.170	0.477	1.665	0.322	AA425853
General transcription factor IIB	Ubiquitous factor required for transcription initiation	GTF2B	0.008	9.807	4.922	10.567	1.077	H23978
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	Helicase and ATPase activities, regulate transcription by altering chromatin structure	SMARCA3	0.003	5.975	0.596	2.778	0.465	AA459407
TATA box binding protein	Binds to TFIID which coordinates initiation of transcription by RNA polymerase II	TBP	0.007	7.004	1.368	3.594	0.513	N50549
Nescient helix loop helix 2	Regulates POMC-derived alpha melanocyte stimulating hormone in the adult hypothalamus	NHLH2	0.012	2.172	0.350	1.370	0.631	H29557
Paired-like homeodomain transcription factor 2	Regulates procollagen lysyl hydroxylase gene expression, involved with regulation of prolactin	PITX2	0.032	8.128	2.457	4.526	0.557	T64905
DEAD (Asp-Glu-Ala-Asp) box polypeptide 48	Nuclear matrix protein	DDX48	0.015	2.465	0.422	2.059	0.835	N79030
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase, 68kD)	Essential human splicing factor, RNA-dependent ATPase and a proliferation associated nuclear antigen	DDX5	0.01	5.063	0.271	2.197	0.434	H27564
TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	May participate in basal transcription, serve as a coactivators, for promoter recognition or modify general transcription factors (GTFs), encodes a subunit of TFIID that interacts with TBP	TAF11	0.008	4.939	1.643	2.321	0.470	N92711
High mobility group nucleosomal binding domain 4	Reduces compactness of chromatin fiber thus enhancing transcription from chromatin templates	HMGN4	0.021	3.649	2.111	1.664	0.456	R43217
Myogenic factor 6 (herculin)	Can activate the muscle differentiation program	MYF6	0.038	0.567	0.820	0.615	1.085	AA176491
General transcription factor IIIA	Exact function not known	GTF3A	0.042	0.487	1.062	0.469	0.963	AA456147
Heterogeneous nuclear ribonucleoprotein L	Likely to play a role in formation, packaging, processing and function of mRNA	HNRPL	0.006	0.656	0.678	0.581	0.886	AA398352
Ephrin type-B receptor 6 precursor	Mediate numerous developmental processes, particularly in the nervous system	EPHB6	0.028	0.638	0.679	0.595	0.932	AA609284

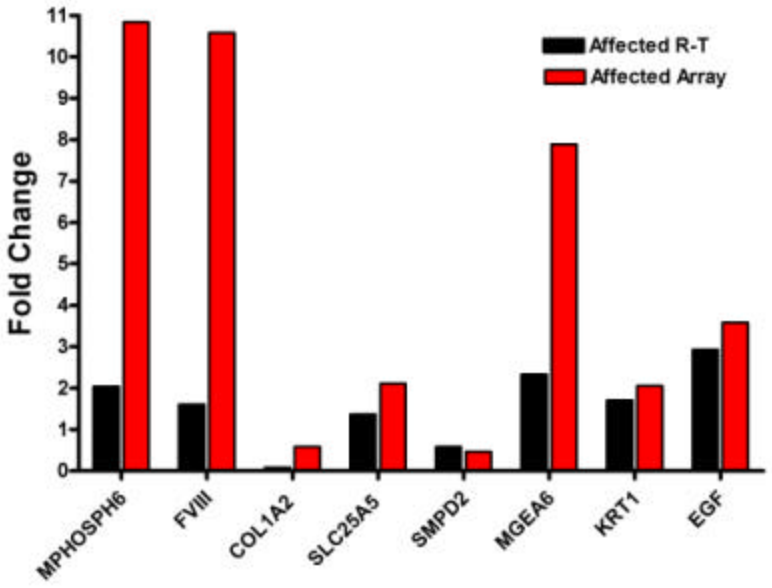
Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Both								
Ligand-dependent corepressor	Widely expressed in fetal and adult tissues, recruited to agonist-bound nuclear receptors	MLR2	0.01		2.426	2.108	0.485	N64794
Chromobox homolog 1 (gene silencing)	Mediates gene silencing	CBX1	0.048		3.102	1.809	0.458	AA448667
Transcription elongation factor B (SIII), polypeptide 1	Activates elongation by RNA polymerase II by suppressing transient pausing of polymerase at many sites	TCEB1	0.002		3.086	2.501	1.086	W81685
Small nuclear ribonucleoprotein D1 polypeptide	Charged protein scaffold to promote SNRNP assembly	SNRPD1	0.027		3.393	2.999	1.093	H16255
Microphthalmia-associated transcription factor	Regulates the differentiation and development of melanocytes	MITF	0.012		1.905	1.695	0.806	N66177
Transcription factor 8 (represses interleukin 2 expression)	Has both transcriptional coactivator and corepressor activity	TCF8	0.02		2.902	2.570	0.599	H46554
Small nuclear ribonucleoprotein polypeptide F	Exact function not known	SNRPF	0.004		2.711	2.087	0.855	AA668189
Nuclear antigen Sp100	Stimulates transcriptional activity of ETS-1	SP100	0.001		4.072	3.692	0.601	AA447482
Nuclear factor (erythroid-derived 2)	Mediates expression of alpha-spectrin gene promoter in erythroid cells in vitro	NRF2	3E-04		0.517	0.449	0.898	H58953
Immune system/inflammation								
Affected								
Leukotriene B4 12-hydroxydehydrogenase	Exact function not known	LTB4DH	0.013	2.796	2.790	1.597	0.998	AA876375
Fc fragment of IgG, low affinity IIIa, receptor for	Upregulated by interferon-gamma in human keratinocytes	FCGR3A	0.031	4.211	2.541	2.940	0.603	H20822
Toll-like receptor 5	Plays a fundamental role in pathogen recognition and activation of innate immunity, expressed in myelomonocytic cells	TLR5	0.012	5.646	2.188	0.912	0.387	N41021
Interferon gamma receptor 1	Encodes the ligand-binding chain (alpha) of the heterodimeric gamma interferon receptor	IFNGR1	0.049	7.002	6.554	6.735	0.936	H11482
CD1C antigen, c polypeptide	May be involved with antibacterial humoral response	CD1C	0.005	5.761	7.201	3.026	1.250	AA002086
Interleukin 13 receptor, alpha 2	IL13 receptor may play important role in early inflammation of psoriasis; however, function is lost in the psoriatic keratinocytes	IL13RA2	0.027	6.138	6.781	6.398	1.105	R52796
Lysozyme	Antimicrobial agent	LYZ	0.036	2.180	1.939	1.163	0.890	AA476274
Interleukin enhancer binding factor 3	Required for T-cell expression of interleukin 2	ILF3	0.043	0.272	0.204	1.250	0.752	R56553
IgG Fc binding protein	Exact function not known	FCGBP	0.005	0.171	0.086	0.625	0.500	T53389
(C-X-C motif) ligand 10	Many effects, including stimulation of monocytes, NK cells and T-cell migration, and adhesion molecule expression	CXCL10	0.038	0.130	0.136	0.715	1.050	AA878880
Interleukin 16 (lymphocyte chemoattractant factor)	Chemoattractant and modulator of T-cell activation	IL16	0.009	0.253	0.195	0.527	0.771	AA454732
CD37 antigen	May play a role in T-cell-B-cell interactions	CD37	0.033	0.097	0.086	1.339	0.887	AA676453
Carrier								
Fc fragment of IgG, low affinity IIb, receptor for (CD32)	Expressed on circulating monocytes, exact function not known	FCGR2B	0.034	5.366	6.855	5.826	1.086	R68106
2'-5'oligoadenylate synthetase 2	Inhibits cellular protein synthesis & viral infection resistance	OAS2	0.018	2.644	1.264	1.759	0.665	R72244
Src-like-adapter	Exact function not known	SLA	0.004	6.086	0.848	2.366	0.389	AA485141

Table 7. Continued

Gene description	Function	Gene name	p-val	Fold?	Normalized values Aff	Carr	Nor	GenBank
Interferon regulatory factor 4	Novel participant in the regulation of lymphoid cell apoptosis	IRF4	0.047	3.576	0.594	1.696	0.474	AA825491
Serum amyloid A1	Produced in inflammatory conditions, role in modulating platelet adhesion	SAA1	0.029	0.453	0.287	0.406	0.898	H25546
interleukin 1 receptor, type I	Important mediator involved in many cytokine induced immune and inflammatory responses	IL1R1	0.03	0.506	0.766	0.496	0.981	AA464526
T-cell receptor, delta diversity 3	Exact function not known	TRDD3	0.028	0.387	0.650	0.362	0.935	AA670107
Major histocompatibility complex, class II, DR beta 1	Central role in the immune system by presenting peptides derived from extracellular proteins	HLA-DRB1	0.046	0.577	0.939	0.591	1.024	AA664195
Advanced glycosylation end product-specific receptor	Receptor for many molecules, inc serum amyloid A, amyloid-beta, S100/calgranulin & advanced glycation end products	AGER	0.033	0.649	0.794	0.606	0.934	W74536
Interferon (alpha, beta and omega) receptor 1	Exact function not known	INFAR1	0.032	0.217	0.415	0.174	0.800	N59150
Miscellaneous								
Affected								
Gephyrin	Anchors inhibitory neuronal receptors to cytoskeleton	GPHN	0.022	5.402	2.798	2.449	0.518	AA779999
Glutamate receptor 2	Predominant excitatory neurotransmitter receptor, activated in a many neurophysiologic processes	HBGR2	0.046	4.679	4.744	1.181	1.014	H06193
Acylphosphatase 2, muscle type	Hydrolyzes the phosphoenzyme intermediate of different membrane pumps	ACYP2	0.005	3.903	3.543	0.747	0.908	N49204
Phenylethanolamine N-methyltransferase	Conversion of norepinephrine; found in basal epidermis and upper dermis	PNMT	0.037	0.344	0.285	0.400	0.828	N63192
Tyrosine hydroxylase	Found in melanocytes	TH	0.018	0.627	0.625	0.984	0.997	AA447751
5,10-methenyltetrahydrofolate synthetase	Regulates folate turnover and accumulation	MTHFS	0.021	0.278	0.126	1.506	0.451	AA777551
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Sulfotransferase activity and a rate-limiting enzyme for the synthesis of heparin	HS3ST1	0.035	0.428	0.225	0.554	0.526	H86812
Synaptogyrin 3	Exact function unknown	SYNGR3	0.02	0.112	0.087	0.520	0.778	N46419
Carrier								
Profilaggrin	Formation of a cornified envelope	FLG	0.046	2.889	3.715	2.416	0.836	AI168528
Biotinidase	Recycles biotin, deficiencies in this enzyme can result in cutaneous disease	BTD	0.013	7.090	1.347	3.262	0.460	R17765
Prodynorphin	Modulates responses to psychoactive substances	PDYN	0.008	7.656	4.137	13.928	1.819	R55796
A disintegrin and metalloproteinase domain 9	TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor	ADAM9	0.005	4.278	1.065	3.714	0.868	H59231
Abnormal beta-hexosaminidase alpha chain	Catalyzes the degradation of the ganglioside GM2, and other molecules containing terminal N-acetyl hexosamines	HEXA	0.042	2.899	1.698	2.190	0.755	T71209
Craniofacial development protein 1	Associated with craniofacial disease, exact function not known	CFDP1	0.017	4.681	0.632	1.786	0.381	AA682613
Alpha-fetoprotein	Fetal counterpart of serum albumin	AFP	0.022	0.538	0.302	0.501	0.931	T59043
Both								
Phosphatidylinositol glycan, class C	GPI lipid anchor biosynthesis	PIGC	0.01		0.273	2.578	0.695	AA007699

A



B

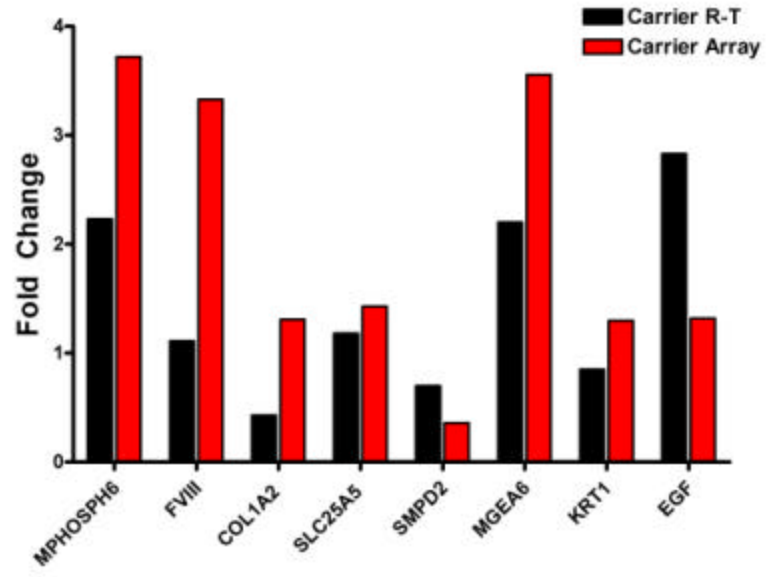


Figure 31. Comparison of fold change in expression levels between quantitative real-time RT-PCR and microarray analysis for the 8 genes selected for array validation for affected (A) and carrier (B) dogs.

Functional annotation of differentially expressed genes of known identity

All genes with known identity were placed in 22 functional categories. Genes with multiple functions that fell into more than one category were classified according to their The magnitude of the differential expression from the microarray analysis was ll genes with known identity were placed in 22 functional categories. Genes with multiple functions that fell into more than one category were classified according to their primary function or most physiologically relevant function. A brief summary of the function of each individual gene is provided in Table 7. In affected dogs, 180 (109 upregulated, 71 downregulated) genes were annotated. Carrier (heterozygous) dogs had approximately the same number of annotated genes at 170 (120 upregulated, 50 downregulated), but a much higher percentage of upregulated genes. Forty-six annotated genes (40 upregulated, 6 downregulated) were differentially expressed in both affected and carrier dogs. The total number of differentially regulated genes as well as the number of upregulated or downregulated genes is reported in Table 8. Figs 32, 33 and 34 graphically display the percentages of up- and downregulated genes in each category for each of the 3 groups (affected, carrier, both). Figure 35 illustrates differences in the total number of genes and the percentage of up- and downregulated genes in each category between affected and carrier dogs.

Table 8. Functional categorization of differentially expressed genes.

Primary functional category	Total	Affected			Carrier			Both		
		Total	Up	Down	Total	Up	Down	Total	Up	Down
Apoptosis, stress, antioxidant	9	7	6	1	1	1	0	1	1	0
Cell adhesion	10	5	4	1	4	3	1	1	1	0
Cell cycle	25	9	5	4	13	10	3	3	3	0
Cell signaling	49	17	12	5	25	18	7	7	6	1
Cytoskeleton and motility	18	11	6	5	6	4	2	1	1	0
DNA repair	15	4	2	2	9	6	3	2	2	0
Endocrine	9	5	1	4	3	1	2	1	1	0
Extracellular matrix	17	6	1	5	10	9	1	1	1	0
RBC-related	14	4	1	3	9	3	6	1	1	0
Hepatic metabolism	10	4	3	1	4	2	2	2	2	0
Immune/inflammatory	23	13	7	6	10	4	6	0	0	0
Intracellular trafficking	15	6	3	3	8	7	1	1	1	0
Ion channel-membrane potential	11	4	2	2	5	4	1	2	2	0
Protein metabolism	11	4	1	3	3	2	1	4	3	1
Protein folding/degradation	23	10	9	1	11	7	4	2	1	1
Protein translation	14	9	8	1	5	4	1	0	0	0
Lipid metabolism	14	10	6	4	2	1	1	2	0	2
Carbohydrate metabolism	8	5	3	2	2	1	1	1	1	0
Energy/respiratory chain	9	5	4	1	4	4	0	0	0	0
Oncogene	20	11	9	2	5	3	2	4	4	0
Transcription	56	23	13	10	24	20	4	9	8	1
Miscellaneous	19	8	3	5	7	6	1	1	1	0
Total		180	109	71	170	120	50	46	40	6

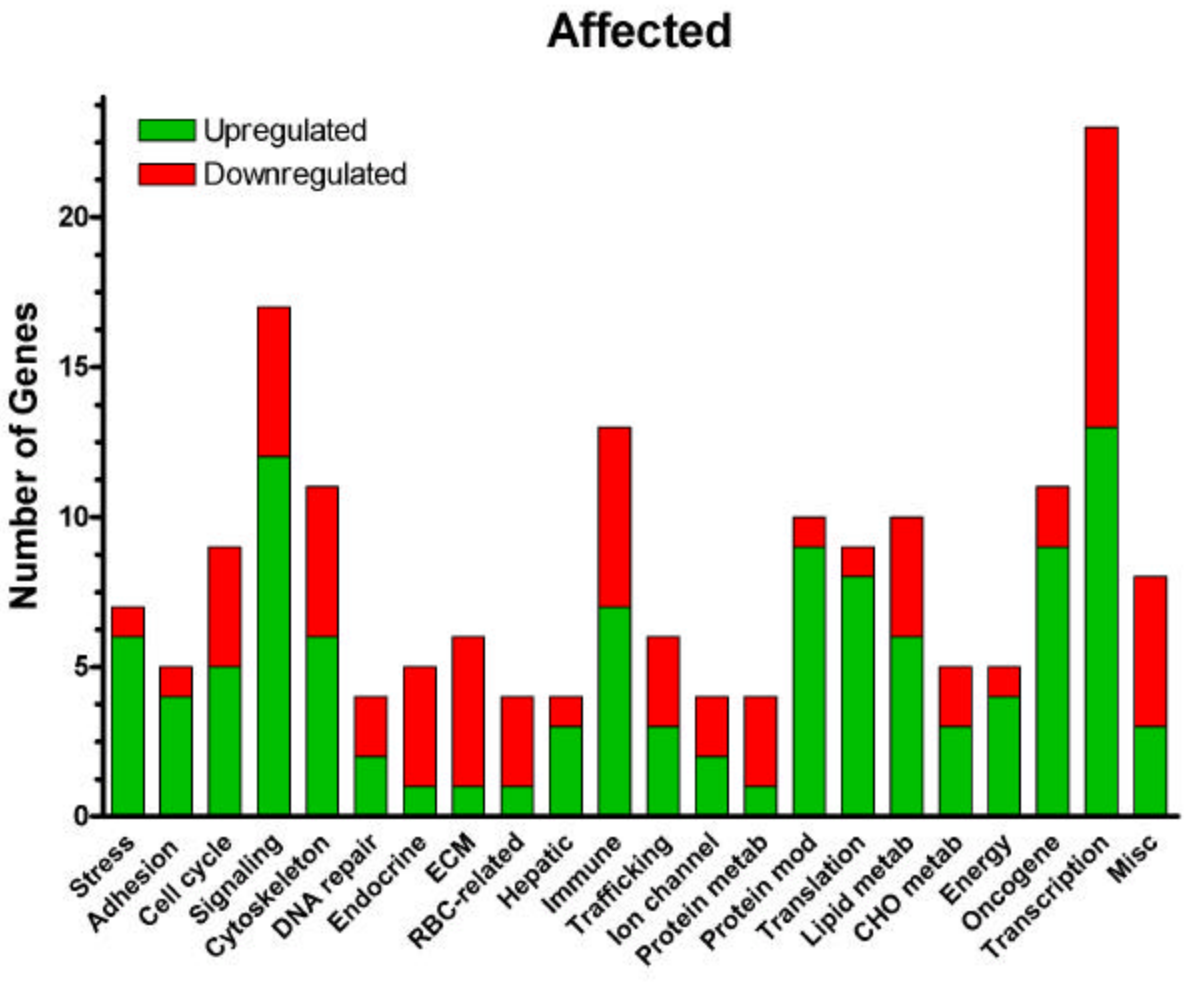


Figure 32. Percentage of genes differentially expressed by category in affected dogs.

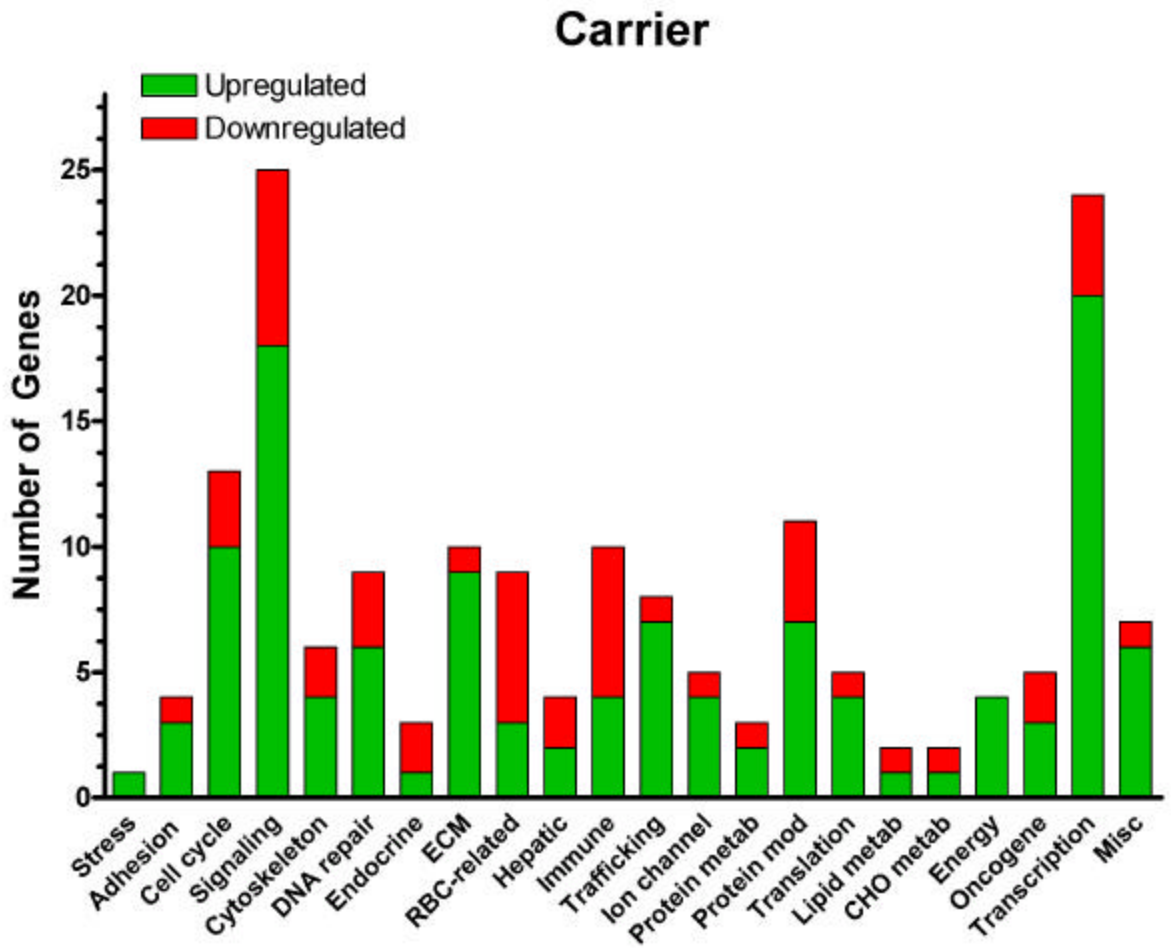


Figure 34. Percentage of genes differentially expressed by category in carrier dogs

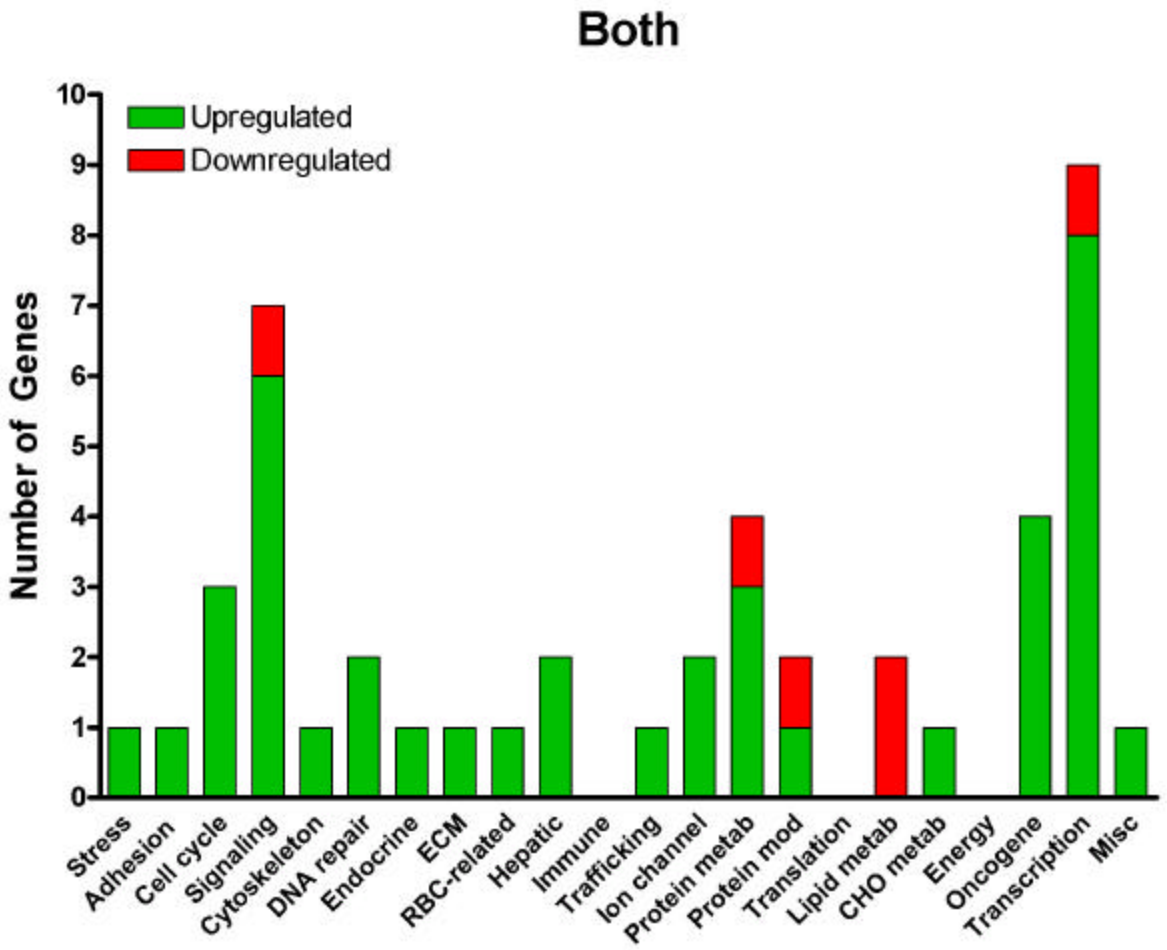


Figure 34. Percentage of genes differentially expressed by category in both affected and carrier dogs.

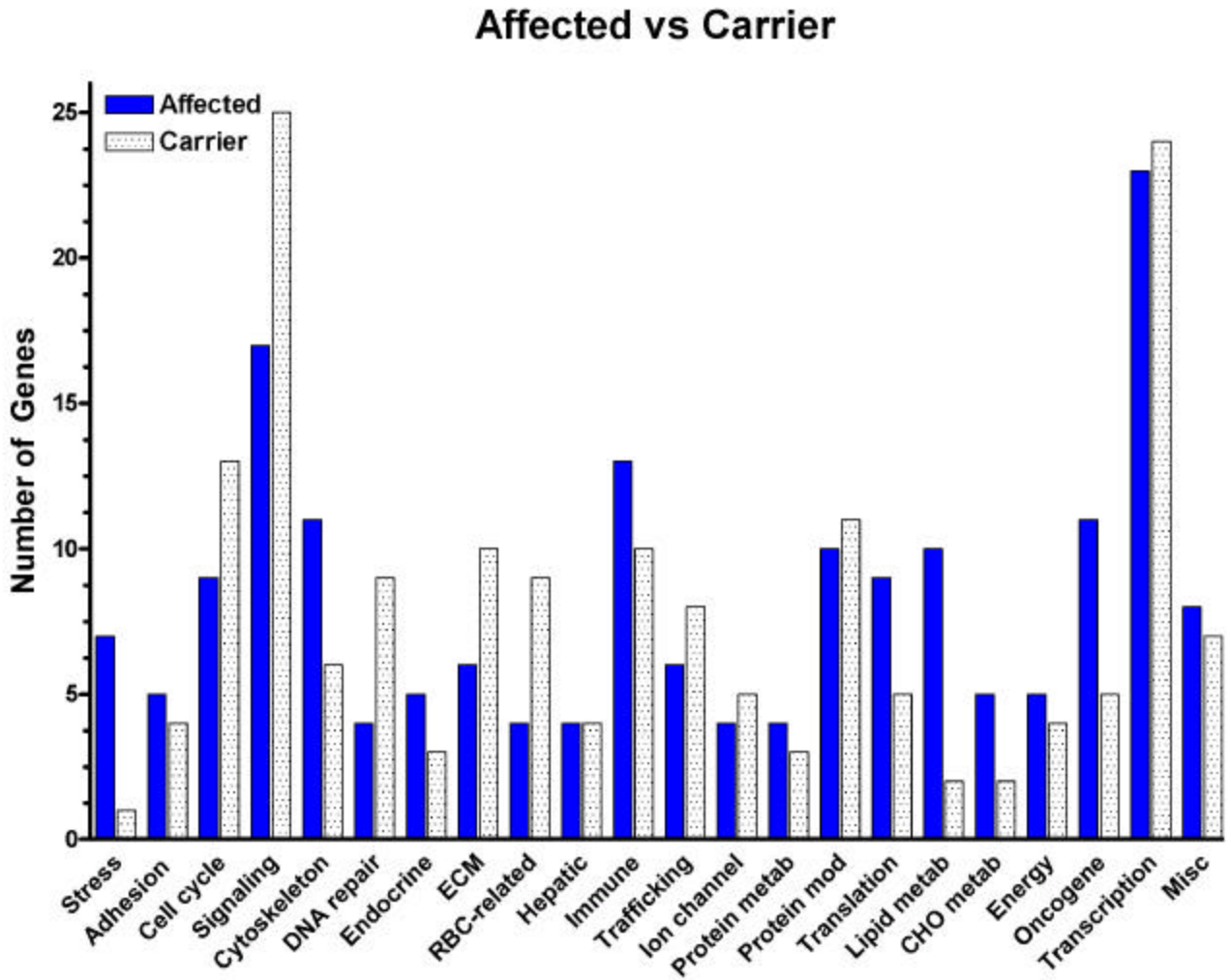


Figure 35. Differentially expressed genes between carrier and affected

CHAPTER VI

SUMMARY/CONCLUSIONS

This dissertation provides a comprehensive analysis of a novel disease caused by a keratin 10 mutation in Norfolk terrier dogs that is termed epidermolytic hyperkeratosis. The clinical and morphologic features are described in detail, and an organotypic cell culture model was established that largely reproduces the morphologic features *in vitro*. The mode of inheritance was defined as autosomal recessive, and a diagnostic test for heterozygotes was developed. Differential gene expression between affected, heterozygous and normal dogs was investigated globally through cDNA microarray analysis. Specific markers of terminal differentiation were also evaluated with semi-quantitative real-time RT-PCR.

Chapter I provides a current detailed review of keratin biology and keratin genodermatoses. The keratin 10 mutation is defined as a point mutation that alters the normal splicing mechanism between exon 4 and intron 5. The consequences of this mutation on splicing and the use of both alternative and cryptic splice sites to produce at least three different transcripts is described in detail. A restriction enzyme-based diagnostic PCR assay was developed for detection of both phenotypically normal heterozygotes and homozygous affected dogs is also described. Finally, a summary of the overall goals and objectives of this dissertation is outlined.

Chapter II contains the first manuscript generated from this work, published in the Journal of Comparative Pathology. This chapter describes in detail the histologic,

immunohistochemical and ultrastructural features of the disease and equates it to epidermolytic hyperkeratosis of humans. Analysis of an extended pedigree over seven generations of dogs confirms the autosomal recessive mode of inheritance.

Chapter III includes the second manuscript generated from this work that is currently under review by Experimental Dermatology. This chapter describes the organotypic culture system established from primary keratinocyte cultures of four normal and two affected Norfolk terrier dogs. The histologic features of both normal and affected cultures at days 7, 14 and 21 are presented.

Cultured epidermis from normal dogs was cornified by day 14 and was markedly hyperplastic at day 21. Morphologically the cultured epidermis was very similar to *in vivo* epidermis with several differences. The stratum corneum of the cultured epidermis was very compact with multifocal areas of parakeratosis in contrast to the loose basket-weave appearance typically seen in skin biopsy samples. The granular cell layer, although not normally very prominent in skin biopsy samples from dogs, was often not apparent. Normal cultured epidermis expressed KRT10 as determined by real-time PCR analysis, but at a reduced level from *in vivo* epidermis.

Cultured epidermis from affected dogs differentiated at a much slower rate than normal epidermis. Cornification was not evident until day 21, and as expected the pattern of cornification was abnormal. The morphologic alterations at day 21 were very similar to those seen in affected epidermis *in vivo*. The suprabasal epidermis displayed prominent vacuolation, and the stratum corneum was convoluted and irregular.

Occasional eosinophilic intracytoplasmic aggregates and rare enlarged keratohyaline granules were noted but not to the extent typically seen *in vivo.*

Chapter IV includes a third manuscript that will be submitted as a concise scientific paper to Veterinary Dermatology. The purpose of this chapter was to validate an alternative internal standard to the "housekeeping gene", GAPDH, for expression studies involving altered keratinocyte differentiation. Traditionally, GAPDH has been used for cutaneous studies, but a large body of research has recently suggested that the use of GAPDH is inappropriate in many experimentally systems, particular the epidermis. Currently, 18S rRNA is widely regarded as one of the most reliable internal standards. The major drawback to the use of 18S rRNA is that it is lost during RNA amplification. For subsequent experiments utilizing amplified RNA, an alternative internal standard needed to be selected. Based on a prior publication indicating cyclophilin as an effective standard for cutaneous studies involving altered differentiation (Steele *et al*, 2002), this gene was investigated through semi-quantitative SYBR green real-time RT-PCR analysis for its suitability. Data obtained from 14 different keratinocyte populations confirmed that cyclophilin is an appropriate standard and is superior to GAPDH.

Chapter V summarizes the data from global gene expression studies using a commercially available cDNA microarray (DermArray, Integriderm Inc., Birmingham, AL) containing sequences for approximately 4400 different genes with known expression in skin. RNA was obtained from 4 normal, 4 heterozygous and 4 affected Norfolk terrier dogs (disease status confirmed through PCR testing) and amplified with a

T7 amplification protocol (MessageAmp, Ambion, TX). Probe labeling was performed with P^{33} radiolabeled dCTP, data were acquired with a phosphorimager, saved as a TIFF file and imported into Pathways 4.0 (Invitrogen) to determine raw intensity and background values. This information was then analyzed through GeneSpring (Silicon Genetics). The data were initially filtered on confidence using the cross-gene error model and then further selected based on a 2.0 fold increase or a 1.5 fold decrease. Based on these methods, 320 (217 up, 103 down) and 298 genes (222 up, 76 down) were considered differentially regulated in affected and heterozygous dogs, respectively. Of these genes, 72 were differentially expressed in the same direction (65 up, 7 down) in both carrier and affected dogs. These genes were then placed in 22 categories according to function.

In conclusion, this work provides a comprehensive phenotypic and genotypic analysis of a recessive form of epidermolytic hyperkeratosis in Norfolk terriers caused by a splice site mutation in keratin 10. This disease is the first reported spontaneous mutation in a superficial keratin in any species and adds significantly to knowledge of keratinization disorders and the function of keratins in the superficial epidermis.

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APPENDIX A

Complete list of the 312 genes that are differentially expressed between normal and both affected and carrier dogs. Of these genes, 212 are upregulated and 100 are downregulated.

Array #	p-value	Fold ?	Affected	Carrier	Normal	Genbank	Name	Description
Upregulated								
824025	0.001	32.930	39.478	4.662	1.199	AA490945	SCAMP1	Secretory carrier membrane protein 1
345430	0.010	23.542	14.195	2.731	0.603	W72473	PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
78217	0.026	18.937	17.726	1.393	0.936	T50699	EBAG9	Estrogen receptor-binding fragment-associated gene 9
360213	0.001	18.396	33.233	11.147	1.806	AA013095	KCNAB1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1
308105	0.020	18.246	17.080	5.408	0.936	N95322		EST
261204	0.023	16.740	15.670	1.793	0.936	H98218	HMGA2	High mobility group AT-hook 2
435948	0.003	16.305	14.266	8.801	0.875	AA701963	AKR1B1	Aldehyde reductase 1
49311	0.003	15.990	38.852	12.005	2.430	H15703		ESTs
128530	0.042	15.240	15.765	1.382	1.034	R10726	SNTB1	Syntrophin, beta 1
785148	0.041	14.459	8.241	2.529	0.570	AA476461	PTPN12	Protein tyrosine phosphatase, receptor-type, zeta polypeptide 1
713862	0.004	14.347	19.673	1.741	1.371	AA284827	KIAA0010	Ubiquitin-protein isopeptide ligase
703707	0.006	13.258	4.702	2.736	0.355	AA278534		ESTs
362680	0.024	13.199	12.481	5.249	0.946	AA018569		KIAA0073 gene product
34641	0.010	12.644	24.818	14.360	1.963	R44404		ESTs
824764	0.025	12.349	19.845	3.158	1.607	AA489050	SENP1	SUMO1/sentrin specific protease 1
32587	0.002	11.723	18.637	6.491	1.590	R43595		ESTs
742767	0.009	11.364	6.117	2.279	0.538	AA400187	NPHP1	Nephronophthisis 1 (juvenile)
859586	0.002	11.280	5.306	3.315	0.470	AA668681	CDC42	Cell division cycle 42
47510	0.031	11.239	9.891	7.836	0.880	H11603	NAPTB	Neuronal adaptin-like protein, beta-subunit

40751	0.0002	11.180	9.862	4.901	0.882	R56219		ESTs
31210	0.011	10.905	13.770	8.440	1.263	R41928	AQP4	Mercurial-insensitive water channel, form 2
784744	0.008	10.827	4.126	1.418	0.381	AA478525	MPHOSP6	M-phase phosphoprotein 6
745360	0.007	10.709	5.381	3.718	0.502	AA625662	MYST1	Histone acetyltransferase 1
31093	0.000003	10.588	13.574	15.389	1.282	R41787	CDH13	Cadherin 13, H-cadherin (heart)
275730	0.004	10.584	6.099	2.329	0.576	R94845	ZNF167	Zinc finger protein 167
758148	0.049	10.579	4.892	1.537	0.462	AA426469	F8	Coagulation factor VIIIc, procoagulant component
897642	0.001	10.160	2.740	1.490	0.270	AA496785	ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1
293940	0.002	10.150	4.648	1.890	0.458	N66043	EEA1	Early endosome antigen 1, 162kD
813552	0.009	10.011	3.528	1.759	0.352	AA455448	CD47	CD47 antigen
452091	0.0002	9.879	11.141	3.376	1.128	AA707148		ESTs
784296	0.034	9.783	6.257	5.224	0.640	AA447079	NR3C2	Mineralocorticoid receptor (aldosterone receptor)
435291	0.042	9.070	3.494	1.889	0.385	AA699908		ESTs
66894	0.003	8.962	5.150	2.174	0.575	T67440	PSARL	Presenilin associated, rhomboid-like
825214	0.029	8.801	3.635	3.253	0.413	AA504113	MPHOSPH10	M phase phosphoprotein 10
281757	0.014	8.736	4.765	1.258	0.545	N48080	GPR88	G-protein coupled receptor 88
739983	0.003	8.378	4.820	3.470	0.575	AA477501	KIF14	Kinesin family member 14
564962	0.034	8.285	5.844	3.913	0.705	AA129397	DAZL	Deleted in azoospermia
781766	0.019	8.159	14.377	6.057	1.762	AA431475	TMEFF1	Transmembrane protein with EGF-like and two follistatin-like domains 1
26811	0.001	8.157	4.887	1.888	0.599	R39148	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
774502	0.008	8.031	9.975	3.776	1.242	AA446259	PTPN9	Protein tyrosine phosphatase, non-receptor type 12
435619	0.026	7.930	3.464	0.975	0.437	AA703191	SERPINA3	Alpha-1-antichymotrypsin
131268	0.014	7.928	3.587	4.482	0.452	R24266	GRB14	Growth factor receptor-bound protein 14
122915	0.037	7.890	8.907	4.024	1.129	T99793	MGEA6	Meningioma expressed antigen 6 (coiled-coil proline-rich)
274638	0.030	7.825	13.928	8.324	1.780	R85414	COD	Carbamoyl-phosphate synthetase 2
436463	0.029	7.823	3.487	1.404	0.446	AA699656		ESTs
1642634	0.019	7.456	5.970	3.961	0.801	AI023804	POLG	Polymerase (DNA directed), gamma 2, accessory subunit
1570311	0.033	7.422	12.814	6.926	1.727	AA932564		KIAA0914 gene product

40017	0.030	7.335	3.811	2.257	0.520	R52654	CYCS	Cytochrome c-1
121857	0.0001	7.318	4.566	2.711	0.624	T97349	SPAG5	Mitotic spindle coiled-coil related protein
79624	0.0002	7.231	5.533	1.776	0.765	T62627	IFI41	Interferon-induced protein 41
281978	0.050	7.173	4.286	1.485	0.598	N51095	RAC3	Ras-related C3 botulinum toxin substrate 3
454543	0.026	7.081	8.982	1.149	1.268	AA677023		ESTs
70030	0.006	7.048	3.050	1.984	0.433	T48761		KIAA1333 gene product
47900	0.049	7.002	6.554	6.735	0.936	H11482	IFNGR1	Interferon gamma receptor 1
361239	0.016	6.848	3.377	2.190	0.493	AA016290	RBBP6	Retinoblastoma-binding protein 6
786084	0.022	6.772	3.102	1.809	0.458	AA448667	M31	Heterochromatin protein p25 beta
784278	0.007	6.772	4.072	3.692	0.601	AA447482	SP100	Nuclear antigen Sp100
32609	0.033	6.745	6.820	11.832	1.011	R43734	LAMA4	Laminin, alpha 4
811942	0.022	6.741	2.780	1.210	0.412	AA455004	GTF2H1	General transcription factor IIH, polypeptide 1
50480	0.019	6.646	5.073	1.672	0.763	H17612	ARG2	Arginase, type II
51448	0.028	6.601	10.257	9.766	1.554	H21042	ATF3	Activating transcription factor 3
282716	0.044	6.491	2.736	2.173	0.421	N49958		EST
1048713	0.048	6.438	4.205	1.118	0.653	AA620638		ESTs
345626	0.006	6.250	2.583	1.211	0.413	W72051	FABP7	Fatty acid binding protein 7, brain
41648	0.027	6.138	6.781	6.398	1.105	R52796	IL13RA2	Interleukin 13 receptor, alpha 2
284799	0.015	6.125	3.331	1.197	0.544	N63107	AKAP79	A kinase (PRKA) anchor protein 5
79520	0.020	6.106	2.490	1.680	0.408	T82415	RABB2	RAB2, member RAS oncogene family
247381	0.022	6.099	14.546	12.431	2.385	N58022	FLJ90650	Laeverin
812105	0.023	6.018	2.470	0.682	0.410	AA456008	AIQ	Transmembrane protein
126674	0.013	5.941	2.890	1.324	0.487	R06909		ESTs
428103	0.005	5.761	7.201	3.026	1.250	AA002086	CD1C	CD1C antigen, c polypeptide
753321	0.003	5.742	2.629	2.245	0.458	AA406589		KIAA0232 gene product
277229	0.012	5.646	2.188	0.912	0.387	N41021	TLR5	Toll-like receptor 5
436431	0.001	5.634	3.726	2.131	0.661	AA699632		ESTs
417508	0.018	5.475	2.186	1.668	0.399	W88655	SULT1C1	Sulfotransferase family 1C, member 1

25584	0.002	5.461	6.915	8.158	1.266	R12802	UQCRC2	Cytochrome bc-1 complex core protein II
291342	0.020	5.423	13.913	8.658	2.565	N72256		ESTs
461988	0.022	5.402	2.798	2.449	0.518	AA779999	GPHN	Gephyrin
767765	0.010	5.363	3.790	2.773	0.707	AA418077	GEM	GTP-binding protein overexpressed in skeletal muscle
27787	0.012	5.283	5.251	3.496	0.994	R40400	CHL1	Cell adhesion molecule with homology to L1CAM
32257	0.031	5.271	9.007	7.560	1.709	R43360	SRP9	Signal recognition particle 9kD
472186	0.016	5.210	3.225	1.091	0.619	AA057378	RAB32	RAB32, member RAS oncogene family
562729	0.004	5.115	5.008	0.977	0.979	AA086471	S100A8	S100 calcium-binding protein A8 (calgranulin A)
448267	0.016	5.036	2.907	1.641	0.577	AA777319		ESTs
148021	0.011	5.007	2.687	1.525	0.537	H13211	CD39	CD39 antigen
284620	0.0001	5.006	2.426	2.108	0.485	N64794	MLR2	ligand-dependent corepressor
281614	0.016	4.978	2.779	2.469	0.558	N48000		Clone DKFZp586L141
50182	0.022	4.975	3.066	0.838	0.616	H17883	KAL1	Kallmann syndrome 1 sequence
296476	0.039	4.889	8.603	5.989	1.760	N74637	PCAF	p300/CBP-associated factor
770957	0.023	4.887	2.037	1.440	0.417	AA430625	DPYD	Dihydropyrimidine dehydrogenase
739155	0.003	4.864	2.272	1.198	0.467	AA421819	CDH6	Cadherin 6, K-cadherin (fetal kidney)
178463	0.015	4.849	2.902	2.570	0.599	H46554	TCF8	Transcription factor 8 (represses interleukin 2 expression)
43622	0.046	4.679	4.744	1.181	1.014	H06193	HBGR2	Glutamate receptor 2
258265	0.018	4.677	2.462	2.274	0.526	N30669	PKIB	Protein kinase inhibitor beta
47142	0.007	4.600	1.807	0.574	0.393	H10965	PEX12	Peroxisomal biogenesis factor 12
120644	0.006	4.589	3.196	2.087	0.696	T95578		ESTs
950507	0.022	4.580	2.510	1.640	0.548	AA599145	ZW10	ZW10 (Drosophila) homolog, centromere/kinetochore protein
258761	0.047	4.556	2.526	1.213	0.554	N30185	XTP1	HBxAg transactivated protein 1
108815	0.020	4.539	2.133	2.280	0.470	T77811	RYK	RYK receptor-like tyrosine kinase
487424	0.021	4.441	2.236	2.304	0.504	AA046724	SYNE1	Spectrin repeat containing, nuclear envelope 1
1388395	0.032	4.393	4.119	1.796	0.938	AA844141	ELK1	ELK1, member of ETS oncogene family
768324	0.005	4.346	3.630	2.469	0.835	AA424807	P44S10	Proteasome regulatory particle subunit p44S10
242062	0.017	4.329	3.735	0.298	0.863	H93332	APOB	Apolipoprotein B (including Ag(x) antigen)

588829	0.046	4.317	1.800	1.088	0.417	AA156571	AARS	Alanyl-tRNA synthetase
788645	0.006	4.283	2.342	1.256	0.547	AA449834	G3BP	Ras-GTPase-activating protein SH3-domain-binding
755385	0.006	4.276	4.292	1.880	1.004	AA410604	CDC16	Cell division cycle 16
51447	0.031	4.211	2.541	2.940	0.603	H20822	FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor for
359287	0.003	4.193	2.214	1.297	0.528	AA016235		ESTs
754275	0.035	4.174	7.879	16.165	1.887	AA479287	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12
81604	0.026	3.982	2.517	1.932	0.632	T65864	AOAH	Acyloxyacyl hydrolase (neutrophil)
340806	0.039	3.927	1.837	0.908	0.468	W56793		ESTs
280252	0.005	3.903	3.543	0.747	0.908	N49204	ACYP2	Acylphosphatase 2, muscle type
24085	0.026	3.867	2.519	1.983	0.651	R39682	TPP2	Tripeptidyl peptidase II
454296	0.005	3.848	1.768	0.359	0.459	AA677149	CLECSF6	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 6
121948	0.008	3.847	3.149	1.210	0.818	T97762	IFRD1	Interferon-related developmental regulator 1
80281	0.020	3.842	2.526	1.689	0.657	T64437		Hypothetical protein BC015088
47559	0.026	3.809	3.363	2.440	0.883	H11455	RAB5A	RAB5A, member RAS oncogene family
742794	0.021	3.804	1.770	0.880	0.465	AA400475	TSPY-like 1	Similar to Testis-specific Y-encoded-like protein 1
769003	0.001	3.766	2.023	1.327	0.537	AA424756		ESTs
115143	0.014	3.757	4.021	5.971	1.070	T86708	SLC4A1	Solute carrier family 4, (anion exchanger), member 1
206052	0.006	3.747	2.697	1.479	0.720	H61552		ESTs
146123	0.018	3.707	6.453	13.342	1.741	R79082	PTPRK	Protein tyrosine phosphatase, receptor type, K
796646	0.006	3.606	3.082	2.373	0.855	AA461467	ODC1	Ornithine decarboxylase 1
306771	0.040	3.585	1.652	1.300	0.461	N91887	TMSNB	Thymosin, beta, identified in neuroblastoma cells
32898	0.033	3.576	1.837	1.160	0.514	R43558	ECHS1	Mitochondrial short-chain enoyl-CoA hydratase
2159880	0.026	3.575	1.639	0.604	0.458	AI480081	EGF	Epidermal growth factor
66532	0.00004	3.521	2.846	2.292	0.808	T67004	EDN3	Endothelin 3
138116	0.024	3.484	2.651	1.999	0.761	R53787	PPP2R5C	Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform
809901	0.001	3.454	3.139	1.282	0.909	AA455157	COL15A1	Collagen, type XV, alpha 1
470187	0.025	3.407	2.419	0.640	0.710	AA029851	GALNT1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
110281	0.040	3.402	1.893	0.754	0.556	T71551	STX7	Syntaxin 7

1292610	0.008	3.388	2.708	1.538	0.799	AA719254		ESTs
878330	0.002	3.386	2.696	1.472	0.796	AA670305		ESTs
897952	0.047	3.380	2.471	1.207	0.731	AA598815	PSMA5	Proteasome (prosome, macropain) subunit, alpha type, 5
786083	0.020	3.312	3.753	2.543	1.133	AA448676	UBE2V2	Ubiquitin-conjugating enzyme E2 variant 2
1469292	0.040	3.306	3.275	2.280	0.991	AA863383	PIM2	Pim-2 oncogene
206907	0.009	3.305	2.277	1.889	0.689	R98695	MEG3	Maternally expressed gene 3
823859	0.014	3.276	3.346	1.717	1.021	AA490466	GJB2	Gap junction protein, beta 2, 26kDa (connexin 26)
418279	0.006	3.270	2.501	2.346	0.765	W90323		ESTs
376802	0.001	3.252	3.351	1.102	1.031	AA047570	PLCD4	Phospholipase C, delta 4
767049	0.031	3.210	3.230	2.087	1.006	AA424503	PSMC6	Proteasome (prosome, macropain) 26S subunit, ATPase, 6
429494	0.006	3.201	2.002	2.264	0.626	AA011347	KOC1	IGF-II mRNA-binding protein 3
852913	0.020	3.169	2.711	2.087	0.855	AA668189	SNRPF	Small nuclear ribonucleoprotein polypeptide F
813611	0.0001	3.122	2.024	0.845	0.648	AA447692		HOX 5.1 gene
137836	0.012	3.107	3.028	1.643	0.974	R68555	TFAR15	Apoptosis-related protein 15
47542	0.040	3.105	3.393	2.999	1.093	H16255	SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide
129616	0.020	3.096	3.538	2.169	1.143	R16656		ESTs
257504	0.002	3.056	2.989	1.879	0.978	N30285	FUSIP1	TLS-associated serine-arginine protein
34255	0.008	3.041	3.029	1.626	0.996	R44202	COMT	Catechol-O-methyltransferase
741880	0.006	2.889	2.622	1.758	0.908	AA403031	PBX1	Pre-B-cell leukemia transcription factor 1
684655	0.034	2.880	2.675	1.497	0.929	AA251770	PSMC2	Proteasome (prosome, macropain) 26S subunit, ATPase, 2
347373	0.015	2.841	3.086	2.501	1.086	W81685	TCEB1	Transcription elongation factor B (SIII), polypeptide 1
1493107	0.013	2.796	2.790	1.597	0.998	AA876375	LTB4DH	Leukotriene B4 12-hydroxydehydrogenase
279966	0.028	2.780	2.488	0.900	0.895	N57551		ESTs
771241	0.040	2.740	4.635	7.205	1.691	AA443587		KIAA0701 gene product
884719	0.019	2.710	2.826	1.686	1.043	AA629567	LDHB	Lactate dehydrogenase B
1471841	0.050	2.637	2.839	1.754	1.077	AA873355	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide
284001	0.002	2.632	2.155	1.728	0.819	N53380	PRKCM	Protein kinase C, mu
282019	0.016	2.611	2.249	1.120	0.861	N48197		EST

897655	0.041	2.532	2.465	1.668	0.974	AA496804	SSFA2	Sperm specific antigen 2
82131	0.007	2.474	2.563	1.395	1.036	T68758	PSMB1	Proteasome (prosome, macropain) subunit, beta type, 1
307660	0.008	2.470	2.759	0.704	1.117	N92901	FABP4	Fatty acid binding protein 4, adipocyte
417263	0.033	2.438	2.089	0.809	0.857	W87781		ESTs
730554	0.041	2.431	2.346	2.361	0.965	AA435940	PEN2	Presenilin enhancer 2
869450	0.025	2.415	2.887	2.072	1.195	AA680244	RBL11	Ribosomal protein L11
789376	0.011	2.395	2.577	1.134	1.076	AA464849	TXNRD1	Thioredoxin reductase 1
267725	0.019	2.389	2.857	2.715	1.196	N25578	BC2	BC-2 protein
815794	0.001	2.374	2.103	0.966	0.886	AA485214	NUCB2	Nucleobindin 2
753917	0.024	2.373	2.413	1.589	1.017	AA479100	P76	76 kDa membrane protein
327350	0.016	2.367	2.684	2.030	1.134	W02101	HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
1499819	0.018	2.367	2.652	1.529	1.120	AA879124	SDS3	Likely ortholog of mouse Sds3
278570	0.049	2.364	1.905	1.695	0.806	N66177	MITF	Microphthalmia-associated transcription factor
278657	0.026	2.343	2.455	2.524	1.048	N62914		EST
271748	0.005	2.333	2.566	1.248	1.100	N31587	RBMS1	RNA binding motif, single stranded interacting protein 1
503155	0.030	2.331	1.647	1.186	0.707	AA148945		ESTs
41511	0.033	2.319	2.296	1.490	0.990	R54097	EIF2B	Translational initiation factor 2 beta subunit
843352	0.027	2.286	2.641	1.386	1.155	AA489343	PSMB7	Proteasome (prosome, macropain) subunit, beta type, 7
545403	0.024	2.272	2.748	2.005	1.209	AA078976	TXNL	Thioredoxin-like
42096	0.005	2.249	2.450	2.224	1.090	R60933	CCT3	Cytoplasmic chaperonin hTRiC5
563444	0.007	2.232	2.415	2.081	1.082	AA112660	FOXF1	Forkhead box F1
884842	0.038	2.225	2.458	1.873	1.105	AA669359	RBL44	Ribosomal protein L44
1642124	0.042	2.223	1.747	1.230	0.786	AI018066	CYLC2	Cylicin, basic protein of sperm head cytoskeleton 2
882511	0.046	2.200	2.415	2.041	1.098	AA676470	M17S2	Membrane component, chromosome 17, surface marker 2
773192	0.038	2.197	2.148	1.951	0.978	AA425687	DDX1	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1
188232	0.047	2.191	2.104	1.674	0.960	H45668	KLF4	Kruppel-like factor 4 (gut)
770674	0.036	2.180	1.939	1.163	0.890	AA476274	LYZ	Lysozyme
842980	0.028	2.175	2.060	1.878	0.947	AA488336	DRG1	Developmentally regulated GTP-binding protein 1

813712	0.048	2.166	2.226	2.058	1.028	AA453765	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
246300	0.026	2.165	1.930	1.846	0.892	N59426	TIA1	Cytotoxic granule-associated RNA-binding protein-like 1
471725	0.032	2.155	4.089	3.186	1.898	AA035450	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1
1474149	0.047	2.151	2.154	1.410	1.002	AA911971	PVRL1	Poliovirus receptor-like 1
380245	0.010	2.146	1.786	1.470	0.832	AA047803	PRKCH	Protein kinase C, eta
815303	0.041	2.119	1.679	0.624	0.792	AA481562	DARS	AspartyltRNA synthetase
796137	0.041	2.118	2.676	2.608	1.264	AA460981	GOLGA4	Golgi autoantigen, golgin subfamily a, 4
897596	0.037	2.112	2.295	1.770	1.087	AA496880	RBL5	Ribosomal protein L5
772304	0.027	2.112	2.391	1.614	1.132	AA404486	SLC25A5	Adenine nucleotide translocator 2 (fibroblast)
971367	0.028	2.112	2.202	0.900	1.043	AA683050	RBS8	Ribosomal protein S8
741769	0.017	2.098	2.134	1.107	1.017	AA402855	POLB	Polymerase (DNA directed), beta
43550	0.028	2.097	2.137	1.252	1.019	H05914	LDHA	Lactate dehydrogenase-A
796513	0.041	2.094	2.487	1.064	1.188	AA460251	NDUFV2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 (6kD, KFYI)
868368	0.039	2.087	2.261	1.873	1.084	AA634103	TSMB4X	Thymosin, beta 4, X chromosome
415145	0.020	2.085	2.124	1.870	1.018	W95082	HSD11B2	Hydroxysteroid (11-beta) dehydrogenase 2
232826	0.010	2.083	2.318	2.079	1.113	H73961	ARPC3	Actin related protein 2/3 complex, subunit 3
50930	0.025	2.083	1.939	1.276	0.931	H19129	FGF12	Fibroblast growth factor 12
469151	0.026	2.062	2.298	1.417	1.115	AA027240	EIF2S2	Eukaryotic translation initiation factor 2, subunit 2beta, 38kDa
379771	0.023	2.053	2.245	1.499	1.094	AA706022	KRT1	Keratin 1
725188	0.049	2.053	2.466	0.842	1.201	AA403295	MDH1	Malate dehydrogenase 1, NAD (soluble)
810383	0.027	2.048	1.999	2.082	0.976	AA464184	EWSR1	Ewing sarcoma breakpoint region 1
786677	0.017	2.016	1.942	1.223	0.963	AA451905	SPATA5L1	Spermatogenesis associated 5-like 1
194384	0.032	2.011	2.312	1.786	1.150	R83000	BTF3	Basic transcription factor 3
625011	0.008	2.006	2.158	0.787	1.076	AA181023	EVI1	Ecotropic viral integration site 1
838568	0.044	2.005	2.019	1.376	1.007	AA456931	COX6C	Cytochrome c oxidase subunit VIc
Downregulated								
711768	0.0002	0.034	0.037	0.167	1.091	AA280832	GALE	Galactose-4-epimerase, UDP-
447568	0.001	0.036	0.044	1.752	1.205	AA702422	MJD	Machado-Joseph disease

824447	0.005	0.040	0.048	1.260	1.201	AA490315	DOCK11	Dedicator of cytokinesis 11
826204	0.007	0.055	0.053	1.054	0.968	AA521453	FLII	Flightless I homolog (Drosophila)
813481	0.006	0.059	0.045	0.637	0.759	AA456069	HOXB13	Homeo box B13
469306	0.005	0.070	0.063	0.502	0.889	AA026118	GRP	Gastrin-releasing peptide
39884	0.012	0.076	0.074	0.835	0.985	R52542	IMPDH1	IMP (inosine monophosphate) dehydrogenase 1
320606	0.003	0.079	0.061	0.198	0.769	W31391	TJP2	Tight junction protein 2 (zona occludens 2)
250069	0.014	0.080	0.058	0.214	0.723	H97140	DUSP8	Dual specificity phosphatase 8
431655	0.033	0.097	0.086	1.339	0.887	AA676453	CD37	CD37 antigen
213136	0.010	0.099	0.093	0.665	0.939	H69583	BTG2	B-cell translocation gene 2
180512	0.010	0.104	0.100	0.302	0.959	R85090	END1	Ectodermal-neural cortex (with BTB-like domain)
279378	0.020	0.112	0.087	0.520	0.778	N46419	SYNGR3	Synaptogyrin 3
769565	0.036	0.124	0.109	1.054	0.875	AA425821	RER1	Similar to S. cerevisiae RER1
415870	0.034	0.129	0.142	0.610	1.106	W86216	ERF	Ets2 repressor factor
825369	0.049	0.129	0.140	0.547	1.085	AA504515		KIAA0121 gene product
1493160	0.038	0.130	0.136	0.715	1.050	AA878880	CXCL10	(C-X-C motif) ligand 10
1553998	0.034	0.133	0.137	0.404	1.032	AA933077	TGFA	Transforming growth factor, alpha
454333	0.049	0.135	0.076	0.464	0.564	AA677254	CD5L	CD5 antigen-like
813644	0.028	0.135	0.087	0.659	0.648	AA447734	PB1	Polybromo 1
810017	0.007	0.139	0.094	1.067	0.672	AA454879	PLAUR	Plasminogen activator, urokinase receptor
742064	0.049	0.141	0.084	0.118	0.593	AA405748	U2AF59	Large subunit of splicing factor U2AF
454317	0.043	0.148	0.104	0.614	0.700	AA677165	AZGP1	Alpha-2-glycoprotein 1, zinc
44692	0.012	0.156	0.069	0.669	0.442	H07089	CRHR1	Corticotropin releasing hormone receptor 1
68767	0.005	0.171	0.086	0.625	0.500	T53389	FC(GAMMA) BP	IgG Fc binding protein
713213	0.016	0.179	0.152	0.318	0.845	AA283631		Homo sapiens chromosome 19, fosmid 39554
221828	0.025	0.185	0.100	1.345	0.538	H92234	KIF1A	Kinesin family member 1A
451711	0.050	0.187	0.153	0.547	0.817	AA707661	ATF6	Activating transcription factor 6
471266	0.011	0.201	0.192	0.881	0.956	AA033564	DGCR6	DiGeorge syndrome critical region 6
221092	0.001	0.203	0.116	0.583	0.571	H91651	GABPB2	GA-binding protein transcription factor, beta subunit 2

795965	0.012	0.204	0.131	0.666	0.643	AA461048	IMPDH1	Creatine kinase, mitochondrial 2 (sarcomeric)
950682	0.007	0.213	0.156	2.513	0.732	AA608558	PFKP	Phosphofructokinase, platelet
453107	0.040	0.223	0.166	0.379	0.746	AA700904	CDC45	Cell division cycle 45
810117	0.002	0.234	0.148	0.887	0.635	AA464982	ANXA11	Annexin A11
725672	0.018	0.238	0.210	0.260	0.880	AA394130	SAZD	Similar to beta-transducin superfamily proteins
813179	0.047	0.243	0.182	0.502	0.750	AA456321	ILGF1	Insulin-like growth factor 1 (somatomedin C)
341588	0.046	0.247	0.179	0.428	0.723	W58368	EIF2B3	Eukaryotic translation initiation factor 2B, subunit 3, gamma, 58kDa
1895835	0.028	0.253	0.228	0.667	0.901	AI292126	SUR1	Sulfonylurea receptor
809776	0.009	0.253	0.195	0.527	0.771	AA454732	IL16	Interleukin 16 (lymphocyte chemoattractant factor)
740620	0.013	0.264	0.217	0.863	0.821	AA477400	TPM2	Tropomyosin 2 (beta)
40781	0.043	0.272	0.204	1.250	0.752	R56553	ILF3	Interleukin enhancer binding factor 3
470122	0.048	0.273	0.111	1.458	0.408	AA029299	KCNMB1	Potassium large conductance calcium-activated channel, subfamily M, beta member 1
448432	0.021	0.278	0.126	1.506	0.451	AA777551	MTHFS	5,10-methenyltetrahydrofolate synthetase
190491	0.046	0.289	0.134	0.980	0.463	H37774	TSC2	Tuberous sclerosis 2
141495	0.027	0.302	0.328	0.539	1.086	R73584	SULT2B1	Sulfotransferase family 2B, member 1
789357	0.026	0.312	0.158	0.705	0.505	AA451716	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
245990	0.046	0.314	0.234	0.687	0.745	N55459	MT1F	Metallothionein (MT)I-F
289857	0.037	0.344	0.285	0.400	0.828	N63192	PNMT	Phenylethanolamine N-methyltransferase
1505469	0.011	0.357	0.376	0.734	1.053	AA905976	PLOD3	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3
813965	0.015	0.366	0.375	0.694	1.025	AA455632		Human chromosome 3p21.1 gene sequence, complete cds
448673	0.001	0.375	0.161	0.576	0.430	AA777375		ESTs
1504731	0.038	0.383	0.376	0.866	0.983	AA905771		ESTs
1572233	0.019	0.384	0.365	0.858	0.952	AA931758	G0S2	G0S2 protein
179890	0.032	0.386	0.289	1.420	0.747	H51574	ALOX5	Arachidonate 5-lipoxygenase
83083	0.039	0.389	0.370	0.362	0.950	T67884	ITIH3	Pre-alpha (globulin) inhibitor, H3 polypeptide
429448	0.043	0.392	0.273	2.578	0.695	AA007699	PIGC	Phosphatidylinositol glycan, class C
50295	0.003	0.394	0.265	1.213	0.672	H17950	HSPA12A	Heat shock 70kDa protein 12A
43771	0.022	0.399	0.378	0.770	0.948	H05655		ESTs

855521	0.012	0.408	0.400	0.599	0.980	AA664179	KRT18	Keratin 18
774446	0.039	0.415	0.162	0.597	0.390	AA446120	ADM	Adrenomedullin
220372	0.035	0.428	0.225	0.554	0.526	H86812	HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1
814576	0.003	0.440	0.451	0.722	1.024	AA480880	ZFP26L2	Butyrate response factor 2 (EGF-response factor 2)
1412344	0.023	0.449	0.404	0.359	0.899	AA844930	GP	Glycoprotein 2 (zymogen granule membrane)
155768	0.003	0.452	0.492	0.669	1.087	R72097	PGA	Pepsinogen A
838856	0.026	0.456	0.498	0.911	1.092	AA481780	CA3	Carbonic anhydrase III, muscle specific
564621	0.002	0.459	0.225	1.189	0.490	AA115877	PI12	Protease inhibitor 12 (neuroserpin)
471498	0.030	0.460	0.380	0.642	0.826	AA035347	GNS	Glucosamine (N-acetyl)-6-sulfatase
248463	0.022	0.460	0.441	0.721	0.959	N59636	HBAZ	Hemoglobin, zeta
433170	0.021	0.464	0.417	0.327	0.898	AA680132	SMPD2	Sphingomyelin phosphodiesterase 2, (neutral sphingomyelinase)
361204	0.041	0.467	0.433	0.723	0.927	AA017526	COL9A3	Collagen, type IX, alpha 3
257135	0.049	0.476	0.383	1.093	0.806	N26836	SLC22A4	Solute carrier family 22 (organic cation transporter), member 4
781342	0.028	0.476	0.477	0.650	1.001	AA448390	C9ORF89	Chromosome 9 open reading frame 89
129146	0.031	0.479	0.289	0.817	0.603	R10896	COX7A2L	Cytochrome c oxidase subunit VII-related protein
487429	0.048	0.503	0.456	0.959	0.906	AA046525	COL6A1	Collagen, type 6, alpha 1
773246	0.036	0.506	0.476	0.534	0.942	AA425772	RFP1	Ring finger protein 1
1031203	0.002	0.512	0.499	0.985	0.973	AA609982	NCYM	DNA-binding transcriptional activator
714472	0.002	0.516	0.480	0.377	0.931	AA293314	RUTBC1	RUN and TBC1 domain containing 1
49591	0.018	0.522	0.419	0.521	0.803	H15155	STS	Steroid sulfatase (microsomal)
712916	0.015	0.523	0.466	0.608	0.892	AA282230	PSMC3	Proteasome (prosome, macropain) 26S subunit, ATPase, 3
451871	0.028	0.525	0.495	0.433	0.943	AA706935	EXTL3	Exostoses (multiple)-like 3
1475595	0.035	0.526	0.473	0.577	0.899	AA873885	ALPL	Alkaline phosphatase, liver/bone/kidney
854899	0.037	0.550	0.544	0.525	0.988	AA630374	DUSP6	Dual specificity phosphatase 6
395539	0.022	0.560	0.231	1.922	0.412	AA757522		EST
30850	0.004	0.561	0.527	1.168	0.940	R42600	MMP17	Matrix metalloproteinase 17 (membrane-inserted)
365973	0.047	0.564	0.490	0.444	0.869	AA063637	PPT1	Palmitoyl-protein thioesterase
160838	0.037	0.568	0.581	0.568	1.022	H24688	SMARCA3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2

207794	0.047	0.576	0.517	0.449	0.898	H58953	NRF2	Nuclear factor (erythroid-derived 2)
811046	0.008	0.578	0.553	0.711	0.956	AA485427	CRIP2	Cysteine-rich protein 2
839991	0.021	0.581	0.616	1.384	1.060	AA490172	COL11A2	Collagen, type I, alpha 2
1420499	0.027	0.582	0.533	0.336	0.915	AA829294	FLJ21908	Hypothetical protein FLJ21908
859422	0.006	0.588	0.533	0.580	0.906	AA666180	ERBAL2	v-erb-a avian erythroblastic leukemia viral oncogene homolog-like 2
74119	0.009	0.591	0.588	0.608	0.995	T54926	SNRPN	Small nuclear ribonucleoprotein polypeptide N
264117	0.005	0.604	0.544	1.028	0.900	N20475	CTSD	Cathepsin D (lysosomal aspartyl protease)
530035	0.046	0.606	0.600	0.394	0.991	AA070489	S100A13	S100 calcium-binding protein A13
383089	0.035	0.607	0.626	0.616	1.031	AA074148	PDE6G	Phosphodiesterase 6G, cGMP-specific, rod, gamma
726768	0.004	0.613	0.625	0.826	1.019	AA398366	SH3GL1	SH3-domain GRB2-like 1
770588	0.016	0.623	0.573	0.361	0.919	AA434139	TIP20	TTF-I interacting peptide 20
813654	0.018	0.627	0.625	0.984	0.997	AA447751	TH	Tyrosine hydroxylase
49873	0.046	0.634	0.584	0.704	0.920	H28922		KIAA0362 gene product
843321	0.015	0.665	0.656	0.526	0.987	AA485959	KRT7	Keratin 7

APPENDIX B

Complete list of the 280 genes that are differentially expressed between normal and phenotypically normal heterozygote dogs. Of these genes, 205 are upregulated and 75 are downregulated.

Array #	p-value	Fold ?	Affected	Carrier	Normal	Genbank	Name	Description
Upregulated								
306921	0.004	22.983	8.284	24.028	1.045	N91962	EEF1E1	Eukaryotic translation elongation factor 1 epsilon 1
31093	0.000001	12.004	13.574	15.389	1.282	R41787	CDH13	Cadherin 13, H-cadherin (heart)
32609	0.006	11.703	6.820	11.832	1.011	R43734	LAMA4	Laminin, alpha 4
435948	0.006	10.058	14.266	8.801	0.875	AA701963	ALDR1	Aldehyde reductase 1 (low Km aldose reductase)
567265	0.006	9.944	1.390	7.302	0.734	AA130633	SRB7	Suppressor of RNA polymerase B, yeast homolog
131268	0.013	9.906	3.587	4.482	0.452	R24266	GRB14	Growth factor receptor-bound protein 14
51362	0.008	9.807	4.922	10.567	1.077	H23978	GTF2B	General transcription factor IIB
878676	0.014	9.328	1.800	3.561	0.382	AA775355	XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5
142331	0.002	9.319	2.724	4.127	0.443	R70546		ESTs
1055766	0.002	9.155	1.682	3.851	0.421	AA628233	P450AROM	Aromatase cytochrome P-450
47510	0.008	8.903	9.891	7.836	0.880	H11603	NAPTB	Neuronal adaptin-like protein, beta-subunit
203351	0.003	8.796	3.759	15.189	1.727	H54289	BEY1	Golgi vesicular membrane trafficking protein p18
754275	0.008	8.564	7.879	16.165	1.887	AA479287	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12
760231	0.034	8.392	1.159	2.762	0.329	AA425628	USP9X	Ubiquitin specific protease 9, X chromosome
739983	0.003	8.378	4.820	3.470	0.575	AA477501	KIF14	Kinesin family member 14
784296	0.0004	8.168	6.257	5.224	0.640	AA447079	MLR	Mineralocorticoid receptor (aldosterone receptor)
66731	0.032	8.128	2.457	4.526	0.557	T64905	PITX2	Paired-like homeodomain transcription factor 2
810791	0.0002	8.110	0.560	5.090	0.628	AA481759	MNAT1	Menage a trois 1 (CAK assembly factor)
767851	0.016	8.054	0.393	3.969	0.493	AA418674	FBN1	Fibrillin 1
427657	0.021	8.018	1.929	3.041	0.379	AA002153		ESTs
825214	0.004	7.875	3.635	3.253	0.413	AA504113	MPHOSPH10	M phase phosphoprotein 10

34149	0.005	7.850	3.448	7.159	0.912	R44762	TTC9	Tetratricopeptide repeat domain 9
145001	0.009	7.758	2.264	16.075	2.072	R78735		ESTs
146123	0.002	7.665	6.453	13.342	1.741	R79082	PTPRK	Protein tyrosine phosphatase, receptor type, K
40692	0.008	7.656	4.137	13.928	1.819	R55796	ENKB	Enkephalin B
782406	0.004	7.611	1.944	6.031	0.792	AA431414	ADSS	Adenylosuccinate synthase
745360	0.002	7.401	5.381	3.718	0.502	AA625662	MYST1	Histone acetyltransferase 1
134748	0.030	7.209	0.969	2.831	0.393	R28294	GCSH	Glycine cleavage system protein H
461354	0.006	7.179	3.899	16.819	2.343	AA704894		Prolactin receptor gene
25517	0.013	7.090	1.347	3.262	0.460	R17765	BTD	Biotinidase
42576	0.0001	7.059	2.067	3.409	0.483	R61332	UBE1	Ubiquitin-activating enzyme E1 (UBE1) mRNA, complete cds
362853	0.002	7.059	3.094	5.445	0.771	AA019459	PTK9	Protein tyrosine kinase 9
859586	0.001	7.047	5.306	3.315	0.470	AA668681	CDC42	Cell division cycle 42
280735	0.007	7.004	1.368	3.594	0.513	N50549	TBP	TATA box binding protein
1326920	0.006	6.972	6.891	9.797	1.405	AA757351	CALCRL	Calcitonin receptor-like
366100	0.004	6.914	9.134	16.646	2.408	AA071473	MATN2	Matrilin 2
1492230	0.019	6.912	1.243	4.728	0.684	AA875933	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1
32493	0.003	6.898	0.564	3.055	0.443	R43483	ITGA6	Integrin, alpha 6
810506	0.003	6.884	9.019	14.297	2.077	AA464529	STK3	Serine/threonine kinase 3 (Ste20, yeast homolog)
1475730	0.002	6.844	2.837	7.139	1.043	AA872690	CCT6A	Chaperonin containing TCP1, subunit 6A (zeta 1)
823562	0.023	6.620	9.020	16.040	2.423	AA497033	CDO1	Cysteine dioxygenase, type I
856535	0.005	6.597	7.138	7.994	1.212	AA633577	MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent)
856489	0.012	6.570	1.629	6.242	0.950	AA633549	RRM1	Ribonucleotide reductase M1 polypeptide
141762	0.031	6.557	2.363	4.033	0.615	R69790	C4ORF15	Chromosome 4 open reading frame 15
267151	0.004	6.549	0.948	3.765	0.575	N23952		ESTs
25584	0.001	6.442	6.915	8.158	1.266	R12802	UQCRC2	Cytochrome bc-1 complex core protein II
740027	0.034	6.423	2.295	2.167	0.337	AA477514	TSNAX	Translin-associated factor X
470261	0.049	6.392	1.872	10.116	1.583	AA028921	SMA3	SMA3
785975	0.014	6.212	2.123	3.530	0.568	AA449742	F13A1	Coagulation factor XIII, A1 polypeptide

360213	0.006	6.171	33.233	11.147	1.806	AA013095	KCNAB1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1,
1584628	0.003	6.170	0.479	2.486	0.403	AA972352	PDLIM3	PDZ and LIM domain 3
784278	0.001	6.139	4.072	3.692	0.601	AA447482	SP100	Nuclear antigen Sp100
815774	0.004	6.086	0.848	2.366	0.389	AA485141	SLA	Src-like-adapter
898123	0.032	6.005	1.510	3.705	0.617	AA598487	GART	Phosphoribosylglycinamide formyltransferase
773330	0.016	6.000	1.322	3.045	0.508	AA425450	GPNMB	Transmembrane glycoprotein
810974	0.003	5.975	0.596	2.778	0.465	AA459407	SMARCA3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3
126681	0.028	5.953	2.973	7.078	1.189	R06918		ESTs
898312	0.044	5.927	1.767	2.679	0.452	AA598826	TRAF4	TNF receptor-associated factor 4
745214	0.002	5.837	3.215	2.018	0.346	AA626867	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2
755474	0.012	5.821	1.316	2.237	0.384	AA410636	IARS	Isoleucine-tRNA synthetase
470092	0.018	5.812	0.547	2.446	0.421	AA029283	LARGE	Like-glycosyltransferase NOTE: Symbol and name provisional
365515	0.021	5.664	4.075	7.646	1.350	AA009609	FGF7	Fibroblast growth factor 7 (keratinocyte growth factor)
79022	0.011	5.589	3.323	8.879	1.589	T61948	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
115143	0.024	5.580	4.021	5.971	1.070	T86708	SLC41	Solute carrier family 4, anion exchanger, member 1
727792	0.027	5.577	0.631	1.667	0.299	AA400893	PDE1A	Phosphodiesterase 1A, calmodulin-dependent
730363	0.040	5.512	0.587	2.597	0.471	AA469954		EST
897751	0.002	5.492	9.498	9.536	1.736	AA599008	PKU-alpha	Serine/threonine kinase
321359	0.004	5.481	1.979	3.271	0.597	W32408	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
241788	0.006	5.457	7.141	4.843	0.888	H91714	FGB	Fibrinogen, B beta polypeptide
730146	0.013	5.431	1.379	2.917	0.537	AA412477		EST
138369	0.034	5.366	6.855	5.826	1.086	R68106	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor for (CD32)
741379	0.049	5.359	0.354	2.434	0.454	AA402766	SMP1	Small membrane protein 1
755506	0.0002	5.260	0.602	3.448	0.656	AA419015	ANXA4	Annexin A4
1323591	0.011	5.232	1.019	2.343	0.448	AA858026	PCI	Protein C inhibitor (plasminogen activator inhibitor III)
247381	0.010	5.212	14.546	12.431	2.385	N58022	FLJ90650	Laeverin
1631713	0.038	5.210	1.446	4.460	0.856	AI025015	NEDD5	Neural precursor cell expressed, developmentally down-regulated 5
773254	0.046	5.170	0.477	1.665	0.322	AA425853	SFPQ	Splicing factor proline/glutamine rich

123546	0.023	5.167	4.133	7.241	1.401	R01566		ESTs
121873	0.009	5.127	5.610	6.373	1.243	T97359		ESTs
896921	0.009	5.078	0.730	1.904	0.375	AA779401	ME3	Malic enzyme, NADP+-dependent, mitochondrial
162775	0.010	5.063	0.271	2.197	0.434	H27564	DDX5	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase, 68kD)
797059	0.003	5.032	1.477	4.445	0.883	AA463251	NAPIL3	Nucleosome assembly protein 1-like 3
813552	0.0004	4.990	3.528	1.759	0.352	AA455448	CD47	CD47 antigen
859858	0.017	4.963	1.087	2.295	0.463	AA679454	STAR	Steroidogenic acute regulatory protein
1642634	0.021	4.947	5.970	3.961	0.801	AI023804	POLG2	Polymerase (DNA directed), gamma 2, accessory subunit
306444	0.008	4.939	1.643	2.321	0.470	N92711	TAF2I	TATA box binding protein (TBP)-associated factor, RNA polymerase II, I
462099	0.016	4.872	0.589	2.296	0.471	AA705377	SENP2	SUMO1/sentrin/SMT3 specific protease 2
108815	0.023	4.851	2.133	2.280	0.470	T77811	RYK	RYK receptor-like tyrosine kinase
449034	0.036	4.846	1.202	2.570	0.530	AA777384	CLDN16	Claudin 16
1573305	0.038	4.810	1.884	2.148	0.447	AA953973	SPARTIN	Spastic paraplegia 20
1461048	0.049	4.759	2.212	2.278	0.479	AA890136		ESTs
450455	0.001	4.732	1.234	4.780	1.010	AA682816	TTL	Tubulin tyrosine ligase
190658	0.030	4.720	3.271	6.068	1.286	H38836		ESTs
41658	0.011	4.702	1.430	2.333	0.496	R66426	GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)
450854	0.017	4.681	0.632	1.786	0.381	AA682613	BCNT	Phosphoprotein (Bucentaur)
842968	0.007	4.670	2.391	6.987	1.496	AA488324	BUB1B	Budding uninhibited by benzimidazoles 1, beta
203132	0.035	4.632	1.116	2.275	0.491	H54629	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
897874	0.006	4.578	2.693	7.023	1.534	AA598635		ESTs
123730	0.024	4.472	3.842	5.904	1.320	R01170	SKAP55R	Src kinase-associated phosphoprotein
841093	0.012	4.469	0.720	2.148	0.481	AA486790	CUL1	Cullin 1
361239	0.009	4.440	3.377	2.190	0.493	AA016290	RBBP6	Retinoblastoma-binding protein 6
810325	0.049	4.371	0.582	2.158	0.494	AA464149	IVD	Isovaleryl Coenzyme A dehydrogenase
447299	0.011	4.355	4.772	5.828	1.338	AA702748		ESTs
284620	0.010	4.350	2.426	2.108	0.485	N64794	MLR2	Ligand-dependent corepressor
121857	0.026	4.345	4.566	2.711	0.624	T97349	SPAG5	Mitotic spindle coiled-coil related protein

40017	0.018	4.344	3.811	2.257	0.520	R52654	CYCS	Cytochrome c-1
307873	0.006	4.332	1.159	2.100	0.485	N93024	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4
178463	0.020	4.293	2.902	2.570	0.599	H46554	TCF8	Transcription factor 8 (represses interleukin 2 expression)
49348	0.025	4.281	1.425	1.988	0.464	H15442	MPI	Mannose phosphate isomerase
1292121	0.037	4.278	0.843	2.112	0.494	AA707615		ESTs
204257	0.005	4.278	1.065	3.714	0.868	H59231	ADAM9	A disintegrin and metalloproteinase domain 9 (meltrin gamma)
487445	0.017	4.249	1.373	6.107	1.437	AA046713	RPS3	RPS3, U15a, U15b genes for ribosomal protein S3, U15a and U15b snoRNA
753234	0.011	4.229	2.811	4.057	0.959	AA406372	ZFX	Zinc finger protein, X-linked
50754	0.011	4.143	0.700	1.983	0.479	H18070	MTIF2	Mitochondrial translational initiation factor 2
40136	0.006	4.093	12.264	8.317	2.032	R53951		ESTs
447510	0.010	4.006	2.357	3.796	0.948	AA702243		ESTs
416409	0.017	3.950	0.611	2.751	0.696	W86876		KIAA0320 gene product
786084	0.048	3.949	3.102	1.809	0.458	AA448667	M31	Heterochromatin protein p25 beta
61387	0.033	3.903	3.775	4.158	1.065	T40899		ESTs
626502	0.018	3.895	0.931	1.440	0.370	AA188179	ARPC1B	Actin related protein 2/3 complex, subunit 1A
824025	0.035	3.889	39.478	4.662	1.199	AA490945	SCAMP1	Secretory carrier membrane protein 1
563451	0.005	3.856	0.953	2.086	0.541	AA113347	TLK1	Tousled-like kinase 1
210717	0.002	3.747	0.726	2.023	0.540	H64347	SDC2	Syndecan 2
447786	0.005	3.737	0.289	1.960	0.525	AA702350	AUTS2	autism susceptibility candidate 2
809828	0.049	3.729	1.429	3.294	0.883	AA455521	E2F5	E2F transcription factor 5, p130-binding
429448	0.010	3.710	0.273	2.578	0.695	AA007699	PIGC	Phosphatidylinositol glycan, class C
1393834	0.031	3.700	2.209	5.196	1.404	AA853954	GCNF	Germ cell nuclear factor
291464	0.043	3.698	1.948	3.139	0.849	N72848		ESTs
347434	0.0001	3.690	1.270	1.571	0.426	W81191	TCEB1	Nucleolar autoantigen similar to rat synaptonemal complex protein
31873	0.021	3.649	2.111	1.664	0.456	R43217	NHC	Non-histone chromosomal protein
742607	0.031	3.598	0.286	1.654	0.460	AA400389		ESTs
1358229	0.047	3.576	0.594	1.696	0.474	AA825491	IRF4	Interferon regulatory factor 4
122915	0.009	3.564	8.907	4.024	1.129	T99793	MGEA6	Meningioma expressed antigen 6 (coiled-coil proline-rich)

377314	0.005	3.547	1.369	3.285	0.926	AA054996	CSNK2A2	Casein kinase 2, alpha prime polypeptide
701417	0.020	3.520	7.063	5.641	1.603	AA286774		ESTs
121406	0.011	3.516	0.534	1.795	0.510	T96688	PKNOX1	PBX/knotted 1 homeobox 1
125183	0.005	3.512	1.587	1.848	0.526	R05694	SSB	Single-stranded DNA-binding protein
898262	0.009	3.489	0.953	3.003	0.860	AA598670	UBE1	Ubiquitin-activating enzyme E1
267859	0.004	3.429	1.178	1.556	0.454	N23315	TBC1D7	TBC1 domain family, member 7
251685	0.002	3.265	1.124	2.500	0.766	H96738	CDH11	Cadherin 11 (OB-cadherin, osteoblast)
234237	0.002	3.247	2.011	1.974	0.608	H69335	PIR	Pirin
291062	0.035	3.240	1.073	1.687	0.521	N72113		ESTs
666829	0.001	3.227	1.357	1.844	0.571	AA234982	SGCD	Sarcoglycan, delta
280740	0.013	3.225	5.992	6.414	1.989	N50544	IRLB	C-myc promoter-binding protein
293696	0.041	3.223	0.837	1.804	0.560	N69672	GPLD1	Glycosylphosphatidylinositol specific phospholipase D1
1055769	0.039	3.220	0.556	2.179	0.677	AA628243		EST
898195	0.039	3.138	2.892	2.377	0.757	AA598567	MYEF2	myelin expression factor 2
1435638	0.034	3.083	0.512	1.520	0.493	AA858175	CBFA1	Core-binding factor, runt domain, alpha subunit 1
24085	0.021	3.044	2.519	1.983	0.651	R39682	TPP2	Tripeptidyl peptidase II
897971	0.018	3.004	1.287	3.123	1.040	AA598868	COPB	Coatomer protein complex, subunit beta
898062	0.040	3.003	0.772	3.881	1.292	AA598776	CDC20	Cell division cycle 20
109265	0.010	2.992	1.782	1.638	0.547	T81033		ESTs
547058	0.012	2.981	0.615	2.646	0.887	AA082943	CCNG1	Cyclin G1
788196	0.010	2.974	2.165	2.584	0.869	AA453404	PPARBP	PPAR binding protein
768324	0.008	2.956	3.630	2.469	0.835	AA424807	P44S10	Proteasome regulatory particle subunit p44S10
110168	0.042	2.899	1.698	2.190	0.755	T71209	HEXA	Abnormal beta-hexosaminidase alpha chain
1677966	0.046	2.889	3.715	2.416	0.836	AI168528	FLG	Profilaggrin
44605	0.039	2.880	1.735	1.914	0.665	H07132	SMP1	small membrane protein 1
66532	0.037	2.835	2.846	2.292	0.808	T67004	EDN3	Endothelin 3
950690	0.022	2.833	2.096	1.678	0.592	AA608568	CCNA2	Cyclin A2
292212	0.004	2.823	1.089	1.580	0.560	N68159	SMRP	Canalicular multispecific organic anion transporter C

796646	0.0001	2.776	3.082	2.373	0.855	AA461467	ODC1	Ornithine decarboxylase 1
41565	0.011	2.753	1.579	2.544	0.924	R52824	MYCN	v-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived
47542	0.027	2.744	3.393	2.999	1.093	H16255	SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide
429234	0.010	2.684	0.455	1.472	0.548	AA007299	TRIO	Triple functional domain (PTPRF interacting)
155806	0.018	2.644	1.264	1.759	0.665	R72244	OAS2	2'-5'oligoadenylate synthetase 2
132702	0.049	2.595	1.105	2.339	0.901	R27004	P4HB	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, beta polypeptide
971199	0.019	2.509	0.799	1.987	0.792	AA774941	NTRK3	Neurotrophic tyrosine kinase, receptor, type 3
32697	0.039	2.501	0.761	1.868	0.747	R43605	CUTL2	Cut-like 2 (Drosophila)
306358	0.015	2.465	0.422	2.059	0.835	N79030	DDX48	DEAD (Asp-Glu-Ala-Asp) box polypeptide 48
45921	0.024	2.461	0.766	1.662	0.675	H09540		EST
730554	0.007	2.446	2.346	2.361	0.965	AA435940	PEN2	Presiniln enhancer 2
852913	0.004	2.440	2.711	2.087	0.855	AA668189	SNRPF	Small nuclear ribonucleoprotein polypeptide F
789182	0.011	2.437	1.842	1.884	0.773	AA450265	PCNA	Proliferating cell nuclear antigen
127677	0.017	2.415	1.648	1.677	0.695	R09691	TRA2A	Transformer-2 alpha
1610408	0.040	2.346	0.442	1.574	0.671	AA995464	NIPSNAP1	4-nitrophenylphosphatase domain and non-neuronal SNAP25-like 1
1161155	0.003	2.329	0.668	2.223	0.954	AA877595	CDKN2A	Cyclin-dependent kinase inhibitor 2A
347373	0.002	2.302	3.086	2.501	1.086	W81685	TCEB1	Transcription elongation factor B (SIII), polypeptide 1
1469292	0.002	2.302	3.275	2.280	0.991	AA863383	PIM2	Pim-2 oncogene
898138	0.021	2.297	2.031	1.875	0.816	AA598492	UBE2B	Ubiquitin-conjugating enzyme E2B
756452	0.049	2.289	0.328	1.608	0.703	AA482128	PTK2	Protein tyrosine kinase 2
267725	0.001	2.270	2.857	2.715	1.196	N25578	BC2	BC-2 protein
788285	0.009	2.252	1.422	1.980	0.879	AA450009	EDNRA	Endothelin receptor type A
810891	0.013	2.181	0.267	1.756	0.805	AA459289	LAMA5	Laminin, alpha 5
52881	0.012	2.172	0.350	1.370	0.631	H29557	NHLH2	Nescient helix loop helix 2
176606	0.0004	2.167	0.626	1.370	0.632	H45300	NELL2	Nel (chicken)-like 2
767798	0.004	2.141	1.460	1.546	0.722	AA418694	ATX1	Antioxidant protein 1, yeast homolog 1
490819	0.023	2.134	0.355	1.870	0.876	AA122287	GARP	Glycoprotein A repetitions predominant
810383	0.004	2.132	1.999	2.082	0.976	AA464184	EWSR1	Ewing sarcoma breakpoint region 1

284001	0.009	2.111	2.155	1.728	0.819	N53380	PRKCM	Protein kinase C, mu
487761	0.014	2.109	1.529	1.706	0.809	AA045180	CA150	Transcription factor CA150
278570	0.012	2.103	1.905	1.695	0.806	N66177	MITF	Microphthalmia-associated transcription factor
610113	0.000	2.097	1.995	1.811	0.864	AA169814	SNX2	Sorting nexin 2
838802	0.027	2.086	1.081	1.801	0.863	AA457671	P4HA1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha polypeptide 1
246300	0.013	2.071	1.930	1.846	0.892	N59426	TIAL1	TIA1 cytotoxic granule-associated RNA-binding protein-like 1
122762	0.049	2.068	1.367	2.133	1.032	T99055	WNT2	Wingless-type MMTV integration site family member 2
589115	0.015	2.067	0.359	1.554	0.752	AA143331	MMP1	Matrix metalloproteinase 1
796137	0.013	2.064	2.676	2.608	1.264	AA460981	GOLGA4	Golgi autoantigen, golgin subfamily a, 4
50566	0.014	2.059	2.075	2.364	1.148	H16796	KCDT10	Potassium channel tetramerisation domain containing 10
23073	0.032	2.058	3.930	2.894	1.406	R38539	FGF2	Fibroblast growth factor 2 (basic)
815861	0.021	2.048	1.722	1.509	0.737	AA485052	PSMD3	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
42096	0.011	2.041	2.450	2.224	1.090	R60933	CCT3	Cytoplasmic chaperonin hTRiC5
1434948	0.009	2.029	0.513	1.869	0.921	AA857131	Tat-SF1	Tat-SF1
25722	0.006	2.024	0.368	2.350	1.161	R36958	NOPE	Likely ortholog of mouse neighbor of Punc E11
813712	0.028	2.003	2.226	2.058	1.028	AA453765	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
Downregulated								
782275	0.028	0.133	0.111	0.120	0.907	AA431748		EST
239611	0.001	0.203	0.051	0.151	0.743	H79534	HBE1	Hemoglobin, epsilon 1
287687	0.032	0.217	0.415	0.174	0.800	N59150	INFAR1	Interferon (alpha, beta and omega) receptor 1
321723	0.033	0.266	0.701	0.231	0.868	W35203	GNG5	Guanine nucleotide binding protein (G protein), gamma 5
455128	0.042	0.272	0.594	0.268	0.985	AA676797	CCNF	Cyclin F
41647	0.007	0.278	1.985	0.291	1.044	R52794	PTPRT	Protein tyrosine phosphatase, receptor type, T
840325	0.012	0.307	0.305	0.358	1.166	AA485397	UBL4	Ubiquitin-like 4
433170	0.023	0.364	0.417	0.327	0.898	AA680132	SMPD2	Sphingomyelin phosphodiesterase 2, (neutral sphingomyelinase)
844680	0.028	0.387	0.650	0.362	0.935	AA670107	TCRD	T-cell receptor, delta (V,D,J,C)
1553998	0.040	0.391	0.137	0.404	1.032	AA933077	TGFA	Transforming growth factor alpha
1412344	0.017	0.400	0.404	0.359	0.899	AA844930	GP2	Glycoprotein 2

814080	0.013	0.416	0.983	0.416	1.001	AA465353	HDAC1	Histone deacetylase 1
321661	0.018	0.418	0.780	0.386	0.923	W35378	PPP2R5C	Protein phosphatase 2, regulatory subunit B (B56), gamma isoform
154749	0.034	0.426	1.431	0.413	0.969	R55620	DPAGT1	Dolichyl-phosphate N-acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)
79629	0.004	0.434	0.643	0.431	0.994	T62636	CXCR4	Chemokine (C-X-C motif), receptor 4 (fusin)
376785	0.033	0.439	0.396	0.257	0.586	AA047567	PMBP	Progesterone membrane binding protein
119530	0.039	0.444	1.052	0.414	0.934	T94781	KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15
754474	0.013	0.448	0.744	0.400	0.894	AA410310		ESTs
161456	0.029	0.453	0.287	0.406	0.898	H25546	SAA1	Serum amyloid A1
840620	0.009	0.462	0.136	0.500	1.082	AA487921		ESTs
417385	0.033	0.477	0.207	0.460	0.965	W88472		ESTs
1475410	0.043	0.479	0.687	0.459	0.958	AA857429		ESTs
796388	0.042	0.487	1.062	0.469	0.963	AA456147	GTF3A	General transcription factor IIIA
149539	0.048	0.492	0.553	0.408	0.828	H00288	DUSP16	Dual specificity phosphatase 16
45542	0.008	0.500	0.757	0.473	0.946	H08561	IGFBP5	Insulin-like growth factor binding protein 5
207794	0.0003	0.501	0.517	0.449	0.898	H58953	NFE2	Nuclear factor (erythroid-derived 2)
416390	0.011	0.503	0.577	0.478	0.950	W86860	NVL	Nuclear VCP-like
810213	0.030	0.506	0.766	0.496	0.981	AA464526	IL1R1	interleukin 1 receptor, type I
1291963	0.015	0.509	1.749	0.433	0.851	AA707464		EST
379796	0.004	0.510	0.695	0.507	0.993	AA706035		ESTs
1556056	0.030	0.511	0.227	0.502	0.983	AA975388	PRPH	Peripherin
365973	0.007	0.511	0.490	0.444	0.869	AA063637	PPT	Palmitoyl-protein thioesterase
838359	0.012	0.514	0.354	0.540	1.052	AA458785	GUCY1B3	Guanylate cyclase 1, soluble, beta 3
812126	0.034	0.525	0.469	0.516	0.983	AA455338	GYPA	Glycophorin A (includes MN blood group)
428338	0.042	0.529	0.619	0.334	0.631	AA005153	INADL	PDZ domain protein (Drosophila inaD-like)
305408	0.027	0.531	0.832	0.480	0.903	N95073		EST
74537	0.022	0.538	0.302	0.501	0.931	T59043	AFP	Alpha-fetoprotein
240062	0.050	0.541	0.237	0.418	0.771	H82236	SLC141	Solute carrier family 14, member 1
1597388	0.032	0.546	0.334	0.509	0.933	AA973224	NDUFA11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11,

								14.7kDa
726438	0.007	0.548	1.119	0.536	0.980	AA399237		ESTs
1420370	0.037	0.553	0.228	0.519	0.939	AA857035	BLVRB	Biliverdin reductase B
243321	0.034	0.562	0.947	0.548	0.976	H95088	PARG	Poly (ADP-ribose) glycohydrolase
812965	0.018	0.562	0.544	0.575	1.022	AA464600	MYC	v-myc avian myelocytomatosis viral oncogene homolog
773568	0.000	0.563	0.702	0.525	0.932	AA428196		ESTs
611255	0.038	0.567	0.820	0.615	1.085	AA176491	MYF6	Myogenic factor 6 (herculin)
324885	0.027	0.568	0.768	0.529	0.930	W48701	C11ORF4	Chromosome 11 open reading frame 4
289677	0.003	0.572	0.751	0.442	0.772	N59893	CG005	Hypothetical protein from BCRA2 region
127841	0.018	0.574	0.634	0.516	0.898	R08829	PKLR	Pyruvate kinase, liver and RBC
758343	0.050	0.576	1.015	0.473	0.821	AA404286	PPIF	Peptidylprolyl isomerase F (cyclophilin F)
448379	0.005	0.577	0.643	0.504	0.874	AA778206	TNPO2	Transportin 2
855547	0.046	0.577	0.939	0.591	1.024	AA664195	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1
770059	0.002	0.577	0.538	0.548	0.949	AA427561	HSPG2	Heparan sulfate proteoglycan
855755	0.027	0.578	0.745	0.509	0.880	AA663986	FBL	Fibrillarin
1519013	0.002	0.586	0.589	0.494	0.843	AA910981		Hypothetical protein MGC29729
81315	0.013	0.589	0.226	0.215	0.365	T60070	SEC4L	GTP-binding protein homologous to Saccharomyces cerevisiae SEC4
236282	0.009	0.596	0.737	0.541	0.908	H61193	WAS	Wiskott-Aldrich syndrome
322914	0.034	0.597	1.227	0.424	0.710	W45148	ACP1	Acid phosphatase 1, soluble
756502	0.019	0.599	0.688	0.547	0.914	AA443998	NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1
1558965	0.013	0.599	0.324	0.627	1.046	AA917769	CDC2l	Cholinesterase-related cell division controller
1474424	0.024	0.604	0.260	0.550	0.911	AA922710	FAM33A	Family with sequence similarity 33, member A
450152	0.005	0.613	0.341	0.589	0.960	AA703449	MEIS3	Meis (mouse) homolog 3
320509	0.030	0.618	0.753	0.588	0.952	W04674	CYB5M	Cytochrome b5 outer mitochondrial membrane precursor
810063	0.031	0.619	0.276	0.604	0.975	AA455303	GFER	Growth factor, erv1-like
950356	0.010	0.629	0.924	0.660	1.048	AA600173	UBE2A	Ubiquitin-conjugating enzyme E2A
826138	0.010	0.636	0.676	0.637	1.003	AA521337	GAMT	Guanidinoacetate N-methyltransferase
308041	0.007	0.636	0.745	0.532	0.836	N92319	PNUTL1	Peanut (Drosophila)-like 1

1031552	0.028	0.638	0.679	0.595	0.932	AA609284	EPHB6	EphB6
416567	0.027	0.640	0.315	0.663	1.035	W86431	PCI	Protein C inhibitor (plasminogen activator inhibitor III)
1475595	0.020	0.641	0.473	0.577	0.899	AA873885	ALPL	Alkaline phosphatase, liver/bone/kidney
124002	0.023	0.645	1.098	0.626	0.972	R01650		ESTs
346604	0.033	0.649	0.794	0.606	0.934	W74536	AGER	Advanced glycosylation end product-specific receptor
838366	0.049	0.651	0.901	0.661	1.015	AA458779	HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase
731404	0.045	0.652	0.743	0.715	1.097	AA412247		ESTs
726846	0.006	0.656	0.678	0.581	0.886	AA398352	HNRPL	Heterogeneous nuclear ribonucleoprotein L
845363	0.028	0.665	1.089	0.676	1.016	AA644092	NM23A	Non-metastatic cells 1, protein expressed in

APPENDIX C

Complete list of the xxx genes that are differential expressed between normal dogs and both affected and phenotypically normal heterozygote dogs.

Array #	p-value	Fold ?	Affected	Carrier	Normal	Genbank	Name	Description
Upregulated								
360213	0.001	18.396	33.233	11.147	1.806	AA013095	KCNAB1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1,
435948	0.003	16.305	14.266	8.801	0.875	AA701963	AKR1B1	Aldehyde reductase 1 (low Km aldose reductase)
49311	0.003	15.990	38.852	12.005	2.430	H15703		ESTs
703707	0.006	13.258	4.702	2.736	0.355	AA278534		ESTs
362680	0.024	13.199	12.481	5.249	0.946	AA018569		KIAA0073 gene product
31093	0.000001	12.004	13.574	15.389	1.282	R41787	CDH13	Cadherin 13, H-cadherin (heart)
32587	0.002	11.723	18.637	6.491	1.590	R43595		ESTs
32609	0.006	11.703	6.820	11.832	1.011	R43734	LAMA4	Laminin, alpha 4
859586	0.002	11.280	5.306	3.315	0.470	AA668681	CDC42	Cell division cycle 42
47510	0.031	11.239	9.891	7.836	0.880	H11603	NAPTB	Neuronal adaptin-like protein, beta-subunit
40751	0.000	11.180	9.862	4.901	0.882	R56219		ESTs
745360	0.007	10.709	5.381	3.718	0.502	AA625662	MYST1	Histone acetyltransferase 1
131268	0.013	9.906	3.587	4.482	0.452	R24266	GRB12	Growth factor receptor-bound protein 14
784296	0.034	9.783	6.257	5.224	0.640	AA447079	NR3C2	Mineralocorticoid receptor (aldosterone receptor)
754275	0.008	8.564	7.879	16.165	1.887	AA479287	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12
739983	0.003	8.378	4.820	3.470	0.575	AA477501	KIF14	Kinesin family member 14
122915	0.037	7.890	8.907	4.024	1.129	T99793	MGEA6	Meningioma expressed antigen 6 (coiled-coil proline-rich)
825214	0.004	7.875	3.635	3.253	0.413	AA504113	MPHOSPH10	M phase phosphoprotein 10
34641	0.006	7.316	24.818	14.360	1.963	R44404		ESTs
784278	0.001	6.139	4.072	3.692	0.601	AA447482	SP100	Nuclear antigen Sp100
25584	0.002	5.461	6.915	8.158	1.266	R12802	UQCRC2	Cytochrome bc-1 complex core protein II

291342	0.020	5.423	13.913	8.658	2.565	N72256		ESTs
247381	0.010	5.212	14.546	12.431	2.385	N58022	FLJ90650	Laeverin
813552	0.0004	4.990	3.528	1.759	0.352	AA455448	CD47	CD47 antigen
1642634	0.021	4.947	5.970	3.961	0.801	AI023804	POLG	Polymerase (DNA directed), gamma 2, accessory subunit
435291	0.029	4.903	3.494	1.889	0.385	AA699908		ESTs
108815	0.023	4.851	2.133	2.280	0.470	T77811	RYK	RYK receptor-like tyrosine kinase
361239	0.009	4.440	3.377	2.190	0.493	AA016290	RBBP6	Retinoblastoma-binding protein 6
284620	0.010	4.350	2.426	2.108	0.485	N64794	MLR2	ligand-dependent corepressor
768324	0.005	4.346	3.630	2.469	0.835	AA424807	P44S10	Proteasome regulatory particle subunit p44S10
121857	0.026	4.345	4.566	2.711	0.624	T97349	SPAG5	Mitotic spindle coiled-coil related protein
40017	0.018	4.344	3.811	2.257	0.520	R52654	CYCS	Cytochrome c-1
178463	0.020	4.293	2.902	2.570	0.599	H46554	TCF8	Transcription factor 8 (represses interleukin 2 expression)
786084	0.048	3.949	3.102	1.809	0.458	AA448667	M31	Heterochromatin protein p25 beta
824025	0.035	3.889	39.478	4.662	1.199	AA490945	SCAMP1	Secretory carrier membrane protein 1
80281	0.020	3.842	2.526	1.689	0.657	T64437		Hypothetical protein BC015088
115143	0.014	3.757	4.021	5.971	1.070	T86708	SLC4A1	Solute carrier family 4, anion exchanger, member 1
429448	0.010	3.710	0.273	2.578	0.695	AA007699	PIGC	Phosphatidylinositol glycan, class C
1469292	0.040	3.306	3.275	2.280	0.991	AA863383	PIM2	Pim-2 oncogene
418279	0.006	3.270	2.501	2.346	0.765	W90323		ESTs
24085	0.021	3.044	2.519	1.983	0.651	R39682	TPP2	Tripeptidyl peptidase II
66532	0.037	2.835	2.846	2.292	0.808	T67004	EDN3	Endothelin 3
796646	0.0001	2.776	3.082	2.373	0.855	AA461467	ODC1	Ornithine decarboxylase 1
47542	0.027	2.744	3.393	2.999	1.093	H16255	SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide
730554	0.007	2.446	2.346	2.361	0.965	AA435940	PEN2	Presiniln enhancer 2
852913	0.004	2.440	2.711	2.087	0.855	AA668189	SNRPF	Small nuclear ribonucleoprotein polypeptide F
278657	0.012	2.408	2.455	2.524	1.048	N62914		EST
267725	0.019	2.389	2.857	2.715	1.196	N25578	BC2	BC-2 protein
347373	0.002	2.302	3.086	2.501	1.086	W81685	TCEB1	Transcription elongation factor B (SIII), polypeptide 1

563444	0.007	2.232	2.415	2.081	1.082	AA112660		ESTs
813712	0.048	2.166	2.226	2.058	1.028	AA453765	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
284001	0.009	2.111	2.155	1.728	0.819	N53380	PRKCM	Protein kinase C, mu
278570	0.012	2.103	1.905	1.695	0.806	N66177	MITF	Microphthalmia-associated transcription factor
796137	0.013	2.064	2.676	2.608	1.264	AA460981	GOLGA4	Golgi autoantigen, golgin subfamily a, 4
810383	0.027	2.048	1.999	2.082	0.976	AA464184	EWSR1	Ewing sarcoma breakpoint region 1
42096	0.011	2.041	2.450	2.224	1.090	R60933	CCT3	Cytoplasmic chaperonin hTRiC5
Downregulated								
1475595	0.020	0.641	0.473	0.577	0.899	AA873885	ALPL	Alkaline phosphatase, liver/bone/kidney
365973	0.007	0.511	0.490	0.444	0.869	AA063637	PPT1	Palmitoyl-protein thioesterase
207794	0.0003	0.501	0.517	0.449	0.898	H58953	NRF2	Nuclear factor (erythroid-derived 2)
433170	0.021	0.464	0.417	0.327	0.898	AA680132	SMPD2	Sphingomyelin phosphodiesterase 2 (neutral sphingomyelinase)
1412344	0.017	0.400	0.404	0.359	0.899	AA844930	GP	Glycoprotein 2 (zymogen granule membrane)
41647	0.007	0.278	1.985	0.291	1.044	R52794		ESTs
1553998	0.034	0.133	0.137	0.404	1.032	AA933077	TGFA	Transforming growth factor, alpha

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